THE BIOCHEMICAL CONVERSION OF PROGESTERONE TO ORCHIC ANDROGENS

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I. PREFACE

About twenty-six hundred years ago, certain Greek thinkers such as Thales of Miletus were interested in explaining the whole cosmos around them by means of a general and unified philosophical theory. The interest of their speculations centered on the states of matter, that is, solid, liquid, and gas, and they conceived the elements earth, water, and air to be the ideal embodiments of these three recognized states. A fourth state, fire, was added later as the principle of heat and motion and as the substance of the heavenly bodies. Perhaps the fact that these states were related and interconvertible by such processes as boiling, freezing, and condensing, was partly responsible two centuries later for the more unifying concept of Aristotle: Everything is composed of a Prime Matter which <u>was</u> without form and distinctive properties until form was impressed upon it. The concrete development of this concept through the ideas of opposing qualities of heat-cold, moisture-dryness led to the theory that anything could be transformed into anything else. Thus, the stage was set for the practice of alchemy and the many centuries of its widespread influence.

It was inevitable that the failure by the ancient chemists, or alchemists, to transmute baser elements into gold and the prostitution of the general philosophy and practice by the religionists, mystics, and charlatans should render unto the word <u>alchemy</u> the contemned meaning which it has today. And it is a point of some historical pathos that the more sincere and

scientifically inclined Aristotelians and alchemists should have been so "right" in their premises yet so "wrong" in their approaches and techniques, and that it was necessary for the whole philosophy and practice to be discredited in order for science to get a fresh start.

Names, meanings, and philosophies aside, man never really stopped looking for the Prime Matter of Aristotle and today considers that it is Energy. And man did not cease the practice of alchemy, even though as a certain progeny of the procession of ancient alchemists, metallurgists, chemists, physicists, and mathematicians, the modern alchemist works under the title of nuclear physicist.

Today we can base our explanations of the cosmos on Energy and the various types of energy and the particular arrangements and interactions of those forms of energy, one manifestation of which is called and recognized phenomenally as Matter. In consideration of parts of the cosmos, one finds that any Thing at any one time is a unique arrangement, a special association of its own component parts, or a particular changing and interaction of the parts. The Thing "Life" must have such a uniqueness, and one may study this quality by determining the associations, arrangements, and organized changes of the energy and matter - or chemical compounds - of Living Things. In attempting to elucidate such particulars, one finds himself in a field within a field, the inner one being Biochemistry.

It therefore seems justifiable that if one's contribution in the field of Biochemistry is judged adequate, he may then receive proper title in the field of Philosophy. Such is the hope in the presentation of this thesis.

II. INTRODUCTION

Like most problems in other scientific fields, problems in biochemistry do not have a spontaneous generation but arise and receive their definition from the previous work of many people. Furthermore, the answers to many scientific questions often must await the development of new methods and techniques, whether these are borrowed from other fields or worked out specifically for the type of problem at hand. Such has been the case for the problem of this thesis which relates to testicular biosynthesis of testosterone and androstenedione from progesterone.

Introduction to the Problem.

One must go back to ancient history to find the time of germination of the problem of the androgens, for it was then that castration of both men and animals provided the first occasions for observations, empirical though they were, of an endocrinological nature. It was obvious that the testes had something to do with the qualities of maleness, such as pitch of the voice, texture of the skin and hair development, muscular strength, and certain characteristics of personality.

In times more modern it was found that the testes were concerned in some way with the conditioning of such accessory sex organs as seminal vesicles, prostates, copulating organs, etc. It has been one of the important efforts of latter day biological science to study in great detail the manner in which these and other effects are brought about by the testes.

It was an event of great importance in culmination of the efforts of many people over a period of a century and a half when in 1935 (1) a chemical compound was isolated from testis material and prepared in pure crystalline form. It was found that this single chemical species, testosterone, could by injection restore to a castrated animal the gross characteristics of maleness which it had previously lost. It had already been found that other compounds - or androgens - possessed similar properties, but testosterone was the first androgen to be isolated from the testis gland itself, and furthermore, it was found to be more potent than any other compound tested. Thus, testosterone, a steroid, assumed an importance of great dimensions.

The questions of how testosterone is produced and how production is controlled naturally arose. It had already been shown that the pituitary gland has some gonadotropic relationship with the testis by experimentation of removal of the gland (hypophysectomy) and by replacement therapy (2). It was also known that androgens administered either to immature or to mature males caused atrophy of the testes, and Moore (3) was first to suggest that this atrophy was the result of pituitary inhibition or decrease in the production of gonadotropic hormones. It was finally clearly demonstrated that partially purified chemical principles of pituitary origin, protein in nature, were able to affect the male gonad (4). One such preparation, called luteinizing hormone or interstitial cell stimulating hormone, was found to act on the interstitial secretory elements of the testes to effect androgen production as evidenced by the enlargement of the secondary sex glands.

Thus, there has been demonstrated a reciprocal type of relationship

between the gonadotropic hormones of the pituitary and the androgenic hormones of the testis, and there is at hand an explanation for one means by which the organism may control its androgen production. One would like to know what the chemical interactions are of these steroid and protein hormones. However, little is known about the biochemical steps of androgen synthesis, and essentially nothing is known about the biochemical mechanism whereby a gonadotropic hormone influences such synthesis. One can postulate that the gonadotropin acts by speeding up one particular step in androgen synthesis, thereby accelerating the succeeding steps. Other controlling factors are also probably involved. It is of some considerable importance therefore as a possible contribution to the understanding of the total organism to investigate some phase of the mechanisms whereby testis tissue synthesizes androgens.

Introduction to the Methods.

Historically speaking, biochemistry arose and progressed as a specialization of organic chemistry, physiology, and anatomy. In the beginning, these sciences generally dealt with macro amounts of material, large measurements, and gross descriptions. However, when in the latter half of the 19th Century, anatomy earnestly began to make full use of the great improvements in microscopes, microtomes, and fixing and staining methods, the science of histology - a type of microanatomy - was developed generally to the point as we know it today, although more modern aids such as the electron microscope and autography with radioactive isotopes have made great contributions not possible before.

Microchemistry awaited the development of the microbalance, first by

Nernst and later by Kuhlmann. Using a Kuhlmann balance, Pregl (5) at the University of Graz, Austria, had by 1911 "revolutionized" most of the existing macrochemical methods in quantitative analysis of organic compounds by the introduction of equivalent micromethods.

Biochemistry, being particularly dependent on the methods made available to it by the general field of chemistry, received great benefit from the development of the methods of microchemistry and also from those of histology and of histochemistry. However, the beginning of the 20th Century was witnessing the genesis of a challenging specialization in the field of physiology. This specialization became known as Endocrinology, and as its problems progressed to the biochemical level, in company with many other branches of physiology, it became evident that tools other than the microbalance and the microscope would be necessary, and that new methods of isolation and resolution, detection, and mensuration would have to be developed.

For example, when progress had been made to the point where endocrinologically active principles could be isolated as chemical compounds, proof was present for the previous supposition that extremely small amounts of these compounds could effect gross physiological changes. It was known, for example, in the second decade of this century that the amount of androgen present in the genital organs is extremely small, and early attempts of several investigators to obtain pure material from testis tissue were failures. Since it was known that blood and urine of normal males also contained androgenic activity, Butenandt and Tscherning (6) went to this source and with success. In their original work, 15,000 liters of urine yielded

15 mg. of androsterone, although later improvements increased the yield. Similarly, when testosterone was finally isolated from testis tissue (1) about 10 mg. pure material was isolated from 100 kg. of steer testes. With the pure compounds it was then possible to equate the activity of these two androgens to each other, to compare the activity of certain volumes of blood and urine, and to establish some general range of physiological production and concentration.

It was found by the first bioassays, which utilized comb-growth in capons after injections of the androgen, that only 15 micrograms (μg.) of testosterone and 100 μg. of androsterone were sufficient to produce gross physiological changes; later, assays by means of direct inunction of the comb area showed that tenths of a microgram were highly effective! (7) These facts forcast the difficulty of investigation on a practical biochemical basis the problems relating to androgens: from what chemical precursors and by what cellular components are the androgens produced in vivo, what are the factors relating to secretion of androgens, how are they otherwise metabolized, and how are all these processes interrelated and controlled. It was, in fact, about 15 years from the time testosterone was first isolated before information began to be available on the biochemical synthesis of that compound and about 20 years before it could be identified as a product provided by the testis to the circulation via the spermatic vein blood. And testosterone has yet to be identified in the peripheral circulation of any animal!

Two great developments in methods have been responsible for the latter day progress in the biochemical problem of the androgens as well as in many other similar problems. One has been the application of isotopes, and in

particular the use of appropriate compounds labeled with radioactive elements. This has allowed the tracing, identification, and quantification of minute amounts of compounds never before possible. The earliest biochemical studies using isotopes were performed by Hevesy in 1924 who used radioactive lead. Later, after the discovery and production of the isotopes of the common elements of the organic compounds, contributions of Schoenheimer and his colleagues were invaluable in pioneering the application of isotopes to problems of metabolism.

The second great advance in methodology was that made in the recent applications of chromatography. Oddly enough, chromatography has been used in some form or other by biological investigators for almost one hundred years as indicated by the work of Schoenheimer and his student, Goppelsroeder (8), with paper chromatography and of Tswett (9) with column chromatography. Even though amazingly good resolution of such things as plant pigments, alkaloids, and proteins was accomplished by their techniques, the work of such men remained generally unknown for many years. In spite of the fact that Willstätter began in 1910 to use and emphasize Tswett's general methods and that Kuhn and co-workers beginning in 1931 successfully utilized chromatography in the separation of polyene pigments, it was not until 1941-44 that the experiments in separating amino acids by column and paper chromatography by Martin, Consden, Gordon, and Synge (10) provided the fulminating influence which was to send chromatography into almost every biological laboratory in the world! Numerous refinements and variations in chromatographic methods have appeared since that time. In the steroid field the two most widely used and successful systems have been those of Zaffaroni (11) and of Bush (12).

A third important development in methodology might also be mentioned - that of ultraviolet and infrared spectroscopy. Technical developments in this area have made great contribution to the identification and quantification of very small amounts of compounds.

The application of techniques of chromatography and of radioactive chemical compounds and also of ultraviolet spectroscopy has been essential to the problem of this thesis and is the procedural substance thereof. Indeed, nearly all that was known previously about the biosynthesis of testosterone had been learned through the use of either one or both of these two general methods, the proper use of which can hardly be overemphasized.

III. HISTORICAL REVIEW

The biochemical problem selected for this thesis was that of the mechanism of conversion of progesterone to orchic androgens, and more specifically, of the conversion *in vitro* of progesterone to 17α-hydroxyprogesterone, testosterone, and 4-androstene-3,17-dione by testis tissue of the white rat. Special emphasis was to be placed on the cellular fractions and the chemical factors involved in the specific conversion of the 21-carbon (C_{21}) compound to the C_{19} compound, that is, the mechanism by which an immediate precursor is converted to the initially appearing testicular androgen.

Historically, the problem had a three-fold development as related to the following divisions of biology: endocrinological physiology; functional anatomy and histology; and endocrinological biochemistry and biosynthesis. The development of the first two was somewhat contemporary and began rather early; the development of the latter has been within the last fifteen years.

Endocrinological Physiology of the Testis.

Much of the attention paid to the testes by the ancients must have arisen from the observations that castration in both animals and man had a definite effect on the maleness of the one so deprived - that is, on the secondary sex characteristics. The first known experimental demonstration of the hormonal action of the testis was carried out by John Hunter in 1794 (13) when he showed that the vestigial spur of a hen would grow into a

masculine type of spur when transplanted to the leg of a cock. Hunter put forth the theory that the accessory sex organs were dependent on the testis for their normal development and condition. This very early work had little influence and was forgotten until about 55 years later when the now famous Berthold (14) showed that the effects of castration of male birds could be reversed by testicular transplants. Loewy (15) in 1903 was probably the first to observe objective changes in castrated animals following the administration of testis extracts; when injected into young capons, these extracts caused skeletal and comb development typical of the normal male.

The first authentic clinical studies of hypogonadism in the male were published by Tandler and Grosz (Vienna, 1907-1910)(16) who studied a religious sect which practiced castration in young children. The authors concluded that the testes were not only concerned with reproduction but also with somatic growth, fat distribution, and psychic development.

In 1911 Pézard (17) repeated the earlier experiments of Loewy with an important refinement which led him to state the following:

> "Un fait expèrimental nous a permis de vérifier l'hypothèse relative à l'action d'une sécrétion interne testiculaire".

He gave to capons intraperitoneal injections of extracts of testes from cryptorchid pigs, having made the important observations that only the interstitial portion of the testes was developed. He noted that a reversal of the castration effects in the capons occurred but that when the injections were stopped, the castration status returned. From this it was concluded that he had not injected viable cells which would continue to function after cessation of the injections, and that indeed the results were due to the action of an internal secretion of the testis.

These experiments were also the first to record the use of the capon comb as an assay procedure. Later, when placed on a quantitative basis by Gallagher and Koch (18), this method became a major tool in the isolation and identification of the androgens.

The experimental work of Berthold, Loewy, and Pézard, all pointed toward the concept that there was something produced by the testis which could exert its influence at a distance from the gland itself and apparently without the intervention of the nervous system. Evidence for a similar type of behavior by other parts - or glands - of the body was also coming to light.

Extremely important were the contributions in the field of experimental physiology by Claude Bernard (1855) who set forth the idea that all organs liberate special substances into the tissue fluids and thereby assist in the maintenance of an internal environment which conditions the activities of cells and tissues. In the same period, Addison's observations (19) upon the syndrome associated with the deterioration of the human adrenal cortex were remarkably accurate and may be regarded as a great achievement. In 1889 von Mehring and Minkowski (20) reported the results of their classical experiments on the effects of pancreatectomy on urinary and blood sugar.

A very important achievement in experimental physiology was the discovery of secretin by Bayliss and Starling (21) in 1902. Starling (22) himself in 1905 first used the term "hormone" with reference to secretin as a simple derivation from the Greek word hormon which means to excite or to set in motion. He later defined a hormone as "any substance normally produced in the cells of some part of the body and carried by the blood stream to

distant parts which it affects for the good of the body as a whole". This is essentially the sense in which the term is used most widely today.

The experimentations quoted above were in fact the beginning of a new and fairly distinct branch of physiology - endocrinology. The "science" of hormonology, or endocrinology, was actually quite nebulous and inexact, however, until chemical studies progressed sufficiently to allow the isolation and synthesis of some of the internal secretions in order to obtain proof that specific chemical identities were able to exert definite actions in the organism. Furthermore, development of the science was delayed by the inevitable damage done by the over-exuberant (Brown-Sequard!), the opportunists, and the quacks.

Great definition and impetus was given to the field when at the beginning of this present century epinephrine, the hormone of the adrenal medulla, was isolated and identified (23). In 1915 the preparation of crystalline thyroxine from thyroid glands of animals was reported (24). A few years later, the studies of Banting and Best (25), of Macleod (26), and of Collip (27) added conclusive proof to the presumptive evidence already present for the existence of insulin, the first crystalline form of which was obtained by Abel and associates (28) in 1926.

In 1927, Aschheim and Zondek (29) discovered that estrogenic material is excreted in considerable quantity in the urine of pregnant women, and it was from this source that the first sexogen to be isolated, estrone, was obtained. The isolation of the crystalline material was accomplished independently in 1929 by Doisy and co-workers (30) of the St. Louis University School of Medicine and by Butenandt (31) at Göttingen. Estrone was unique in relation to the other hormone-like compounds previously isolated in that

it was a lipid and, indeed, a steroid! The first ovarian estrogen to be isolated was the related steroid, estradiol-17β, this being accomplished some six years later (32).

In 1927 McGee (33), working in Koch's laboratory at the University of Chicago, prepared a very potent androgen extract from bull testis by alcoholic extraction. Profiting from the experience with the estrogens, workers investigated male urine for the presence of androgens, and it was found that androgenic material is indeed excreted in the urine, though in extremely small amounts (34). Four years later the isolation of crystalline androsterone from human urine was accomplished by Butenandt and Tscherning (6) and in 1934 the less active androgen, 5,6-dehydroepiandrosterone, was isolated from the same source (35). Butenandt then synthesized androstane-3α,17β-diol by the reduction of androsterone, and it proved to be about three times as potent as androsterone (36). This fact suggested that androsterone might not be the testis hormone. Furthermore, early in 1935 it was reported by Dingemanse and co-workers (37) that material from testes had about five times the activity per capon unit as androsterone in promoting growth of seminal vesicles in castrate rats. Just previously, Gallagher and Koch (38) had shown that the active principle from testes was unstable to boiling alkali, a reaction not characteristic of urinary androgens. All these facts supported the supposition that the most active testoid factor had yet to be isolated.

About this time three different groups (American, German, and Swiss) (39) reported the synthesis of 4-androstene-3,17-dione from dehydroepiandrosterone. Androstenedione had not been isolated from natural sources, but it was found to be almost as active as androsterone in the capon test and of

particularly high potency in the castrate rat test (40). Androstenedione was known to be similar to the active principle of the testes extracts in its instability to alkali, and so at this stage androstenedione appeared to be similar to the testicular hormone. It was not identical, however, because assays of purified concentrates of testicular extracts were still more potent than androstenedione in both the rat and capon tests.

Finally, in June of 1935, came the report from Laqueur's laboratory (1) of the isolation of a testicular product to which the name testosterone was given. Pure testosterone was found to be about ten times as powerful as androsterone in promoting comb growth and about forty times as potent in the castrate rat. Chemical synthesis by Butenandt and Hanisch (41) from dehydroepiandrosterone and by Ruzicka (42) from the same compound and from cholesterol helped to establish the structure of testosterone as 4-androsten-17β-ol-3-one.

From the time of the isolation of testosterone from bull testes, it was eleven years before it was isolated from a second species. To date, it has been isolated from the testes of the bull, stallion, rabbit, swine, and man (43). Thus, it appears to be a testicular hormone of general occurrence. Other steroids isolated from this gland are the following: 16-androsten-3α-ol and the 3-epimer from hog testis; 5-pregnen-3β-ol-20-one (pregnenolone) and the two allopregnanolone epimers from swine; estrone from stallion, and estradiol-17β from both stallion and human testes. These latter steroids seem not to have any androgenicity, however (43).

For many years, testosterone was generally accepted as the testis hormone. However, the following considerations still had to be made. The finding of a physiologically active compound, however potent, in a tissue does not mean

that it is the compound which the gland secretes into the blood stream for transport to the rest of the body. Second, it may be that the gland also secretes effective amounts of other compounds and that these may be quite physiologically influential even though less active on a molar basis as determined by bioassay. Hence, it is quite important to determine what compounds and what quantities thereof are provided to the total organism by the endocrine organ. Third, it may be that a metabolite of the secretory product may be largely responsible for the hormonal effects rather than the secreted substance itself. Thus, it also becomes important to know the levels of circulating androgens. Eventually, of course, one would like to determine the exact chemical species responsible for effects at the cellular level and thence at the enzymatic level.

To date, testosterone has not been found in peripherally circulating blood. It was finally isolated and identified, however, from the spermatic vein blood of dogs by West and co-workers (44). They obtained about 6 liters of blood by cannulating the spermatic veins of six anesthetized, heparinized dogs. From the serum of this blood, testosterone, androstenedione, and 7-ketocholesterol were isolated and identified by paper chromatography and infrared analysis. Cholesterol and unidentified compounds were also obtained. Unfortunately, full publication of these experiments has not appeared; the amounts of compounds obtained were not given, and one cannot determine the fraction of androgenic potency of the spermatic vein blood provided by testosterone and androstenedione. However, this work definitely established that the two androgens, testosterone and androstenedione, are secreted by the testis. Thus, by definition, they are true hormones.

It should be mentioned that androgens also occur in the urine of the female and that androgens are products not only of the testis but also of the ovary, adrenal cortex, and perhaps the placenta (45). However, the most active androgen, testosterone, seems to be produced only by the testis, although an ovary has been shown to be able to convert testosterone-3-C^{14} to 17β-estradiol-C^{14}. This suggests that testosterone may be an intermediate in the biosynthesis of estradiol (46). Normal production of extra-orchic androgens perhaps plays a rather important role in general body metabolism in both male and female.

Functional Anatomy of the Testes.

Cellular Arrangement for Androgen and Spermatozoa Production:

In 1677 Anton van Leeuwenhoek reported that by using his invention, the microscope, he was able to detect small "animalcules" in semen; this was the first description of human spermatozoa. Schleiden and Schwann in 1839 showed the structural unit of plant and animal organisms to be the nucleated cell, and thereafter spermatozoa were recognized also as true cells. About ten years later, Berthold made his second great contribution by showing that the testis proliferates spermatozoa. In 1865 the Italian histologist Enrico Sertoli discovered the "footcells" to which the spermatids are attached during their development. About the middle of the same century, the German anatomist Franz von Leydig described the interstitial cells of the testis, which now bear his name. When it later became quite clear that the testis is capable of elaborating androgenic chemical compounds, there was considerable interest and argument concerning whether these cells or the cells of the seminiferous tubules produce the testis hormone.

Selye (16) states that in 1922 Lipschütz and Wagner pointed out that in spontaneously eunuchoid rabbits with atrophic accessory sex organs, the Leydig cells were poorly developed, while the seminiferous epithelium remained normal. Hence, they ascribed the role of hormone production to the Leydig cells. (It seems that these observations came about eleven years after similar ones by Pézard concerning the interstitial cells of the cryptorchid testis of the pig!) Bouin and Benoit (47) summarized the evidence for support of such a conclusion in citing experiments which showed that vasoligation, X-ray treatment, exposure to heat, cryptorchidism, and other interventions which destroyed the seminiferous epithelium but left the Leydig cells intact did not interfere with the androgenic activity of the testis. Histologically, the interstitial cells appear to be endocrine in nature; they are large cells occurring singly or in groups, are not drained by ducts, and are relatively near the blood supply since the blood vessels of the testis course through the intertubular spaces, not penetrating the walls of the tubules. It is of interest that the Leydig cells appear in the human embryo and are abundant at birth, then subsequently almost disappear, to return to full activity at the time of puberty (48).

Correlative work which helped further to clarify the endocrine function of the Leydig cells was that done in relation to gonadotropic hormones of the anterior pituitary gland. From the early work of Smith (2) and of Zondek and Aschheim (49) it was deduced that pituitary extracts produced two types of gonadal reactions: first, the stimulation of follicular growth in the ovaries and of spermatogenic activity in the testis; and second, the final ripening of the follicles and production of corpora lutea in the ovary, and in the testis

activation of the Leydig cells. When careful fractionation of the hypophyseal proteins responsible for these responses was carried out, it became clear that the seminiferous epithelium responded mainly to one hormone, the follicle stimulating hormone (FSH), and that the Leydig cell system was stimulated by another, the interstitial cell stimulating hormone (ICSH). When immature or hypophysectomized animals were used, it was further found that manifestations of testis hormone secretion were elicited only by ICSH (50,51,52,53). Thus, the connection between the pituitary gland and the Leydig cells, and the Leydig cells and androgen production was unequivocally demonstrated.

The first known gonadotropin was found in the blood and urine of pregnant women by Ascheim and Zondek (54). At first this principle, now called human chorionic gonadotropin (HCG), was not distinguished from ICSH, the pituitary hormone, but the former was later found to arise from the placenta and to differ from the latter chemically and in certain aspects of its hormonal action (51).

Chorionic gonadotropin was found not to stimulate follicular development in the hypophysectomized female rat but to repair interstitial cells in the hypophysectomized male (51). It maintained seminiferous epithelium in the newly hypophysectomized animal but would not restore the atrophied system. Since HCG induced larger Leydig cells and greater response of the seminal vesicles and prostate, its potency as an interstitial cell stimulating agent appears to be greater than that of ICSH (51). In adult men, normal and hypogonadal, HCG caused the Leydig cells to increase the output of both androgen and estrogen; increased excretion of the latter was more marked and was declared to be a more sensitive and reliable indicator of the response to the hormone (55).

Intracellular Arrangement for Androgen Production:

As the organism is investigated to find the gland, and as the tissue of the gland is investigated to find the cells, so must the components of the cells themselves be sought which are responsible for certain chemical transformations known to take place in the whole cell. The apex of this pyramid is the isolation of the enzymes responsible. Since some studies were carried out to determine cellular components involved in the various steps of androgen synthesis, a limited discussion of such structures and the methods of study thereof is in order.

Cytologists for many years have known that the cell is not a homogeneous mass of protoplasm but that both plant and animal cells contain several discrete structures that can be seen and identified. Three general techniques have been used to study these structures: those of histochemistry (56), the submicro techniques of Linderstrøm-Lang, Holter, and their associates (57), and those of differential centrifugation (58).

The method of differential centrifugation of broken cell suspensions was introduced in 1934 by Bensley and Hoerr (59), who described the isolation of mitochondria from guinea pig liver. Since then, many papers have appeared dealing with the isolation of cellular components, the most recent ones describing procedures for the fractionation of a tissue into nuclei, mitochondria, submicroscopic particles (or microsomes), and soluble material (58,60).

The methods of Hogeboom (60) are exemplary. In the preparation of these particles, liver tissue was placed in cold 0.25 molar sucrose and submitted to homogenization. The broken cell suspension (homogenate) was then centri-

fuged at low speed (ca. 700 x *g*) for 10 minutes to remove unbroken cells, nuclei, some mitochondria, connective tissue, and other jetsam. The remaining material not thrown down (supernatant) was then centrifuged at high speed (ca. 5000 x *g*) for 10 minutes. The sediment obtained by this operation consisted mainly of mitochondria and Golgi apparatus but also of some microsomes. Twice the sedimented material was resuspended and centrifuged at 20,000 x *g*. The final suspension of thrice-sedimented mitochondria was reasonably pure on the basis of cytological criteria, but the biochemical homogeneity of the preparation was open to question.

The preparation of pure mitochondria is not easy, and many results reported in the literature have been later found in error due to the fact that the mitochondrial preparation was contaminated by other material, usually submicroscopic particles. However, it seems fairly well established that all the succinoxidase activity of the rat or mouse liver cell is contained in the mitochondrial fraction and that the mitochondria are mostly but not solely responsible for the reactions of the Krebs cycle (58). Many other enzymes and enzyme systems have been implicated as having a mitochondrial locus. DPN- and TPN-cytochrome c reductase have been found in both mitochondria and submicroscopic particles; catalase has been found to some extent in the mitochondria but by far the greater activity was recovered in the supernatant containing submicroscopic particles and soluble material (58).

The separation of the submicroscopic particles, or microsomes, required centrifugation at very high speeds (54,000 x *g*) for 1 hour (60). The sedimented material consisted of cytoplasmic particulate material too small to be resolved in the optical microscope. The results of several investigations

have indicated that a fraction so obtained is both cytologically and biochemically heterogeneous. One of the very striking properties of this fraction, first suggested by the experiments of Claude (61), is the high concentration of pentose nucleic acid. Less work has been done on the microsomal fraction than on the mitochondrial one, but several enzymatic actions have been attributed to the microsomes. As a result of careful work, Beyer and Samuels (62) have reported that the 3β-ol dehydrogenase system of the beef adrenal cortex (see p. 26) was associated with the microsomal fraction. Systems which reduce the 11-keto group of certain steroids have also been so implicated (63).

It is interesting to note that the lipid content of liver submicroscopic particles is relatively quite high - about 40 per cent of the dry weight, a value considerably higher than that for mitochondria. Most of this lipid is present as phospholipid (58).

It is further quite important to note that the lipid content of testis tissue is quite low but that the Leydig cells (or interstitial cells) are nearly as rich in fatty substances as the adrenal cortex or corpus luteum (16). The principal fatty substances of these androgen-synthesizing cells of the testis have been shown to be fatty acids, cholesterol, cholesterol esters, and phosphatides. It seems consistent, therefore, that certain reactions involving steroids have been found to take place in the lipophilic microsomes of liver and adrenal cortex.

The supernatant remaining after the submicroscopic particles had been sedimented contained all particles smaller than those of about 50 mμ. diameter or all particles having molecular weights less than about 100,000,000 (60).

These were rough approximations because sedimentation at any particular centrifugal force depends on the size, shape, and density of the particle as well as certain properties of the medium. However, the material remaining after very high speed centrifugation was referred to as "soluble". The main components appeared to be proteins, and the solution was enzymatically complex. In determining the enzymatic activity associated with the soluble fraction it is of considerable importance to ascertain that the factors associated with the process have not come from ruptured particles.

It has been reported that in the case of rabbit liver, all the enzymes involved in glycolysis of glucose to lactic acid were present to some extent in the soluble fraction (64). The mitochondria and microsomes of these tissues possessed essentially no glycolytic activity by themselves but produced pronounced stimulation of the supernatant, which had previously been only 50 per cent as active as the whole tissue. This indicated that the particulate fractions probably contained cofactors required by the enzymes of the supernatant.

The Biosynthesis of Androgens.

In 1940 Danby (65) reported net androgen synthesis by surviving bull testicles perfused with blood as indicated by the production of substances which promoted comb growth in chicks. The compounds formed were not identified. After adding homologues of testosterone (dehydroandrosterone, androstenedione or androstenediol) to the perfusion blood, a "surplus of comb growth promoting substances" was present in the perfusion blood and the perfused organ after the experiment. It was considered that the added steroids had been converted into more potent androgens. Gonadotropic substance from human pregnancy urine showed no effect on the formation of male hormone

in vitro.

It was shown in 1950 that slices of testis tissue were able to synthesize cholesterol from C^{14}-labeled acetate (66). Savard and co-workers (67) perfused acetate-1-C^{14} through a human testis, with the result that C^{14}-labeled testosterone and androstenedione were formed. Brady (68) performed experiments showing that testis tissue slices from hog, rabbit, and human were able to synthesize testosterone from C^{14}-labeled acetate. He further found that the addition of chorionic gonadotropin to the incubation medium markedly stimulated the conversion of acetate to testosterone. Such a stimulation was not seen in the conversion of acetate to cholesterol by the same preparations; he thus concluded that testosterone was being synthesized from acetate by some other route than via cholesterol.

The interest in the experiments by Brady in the consideration of cholesterol as a possible precursor of testosterone arose from significant findings in the companion field of adrenal biochemistry. These were that cholesterol is probably a precursor of the corticosteroids, though perhaps not an obligatory one (69,70). Furthermore, evidence has been obtained that one site of action of adrenocorticotropic hormone was in the conversion of C_{27} cholesterol to a C_{21} steroid (71). Thus, there was the question of the role of cholesterol in testosterone production and the relation of gonadotropin to this process.

Though from Brady's experiments cholesterol appeared not to be on the pathway in testis from acetate to testosterone, the evidence began to imply that perhaps 21-carbon steroids are involved. For example, progesterone (72), 16,17-dehydroprogesterone (73) and desoxycorticosterone (74) were found by

bioassay to be weakly androgenic. Pfiffner and North (75) tested 17α-hydroxyprogesterone and found it to possess an activity about equal to that of androsterone on the seminal vesicle and prostate of the immature castrate rat. Butenandt (76) also found that it was somewhat active in castrate rats and when applied to the chick comb. Dorfman (73) determined, however, that the comb response was not proportional to dose; low and high dosages were inactive while a medium dosage (320 μg. over a seven day period) was active. When 17α-hydroxyprogesterone was injected subcutaneously at a dosage of 1000 μg., a significant comb inhibition was observed. Thus, Dorfman's results with 17α-hydroxyprogesterone were rather equivocal. Del Greco et al. (77) found that feeding 17-hydroxysteroids to female rats gave an increase in urinary 17-ketosteroids whereas compounds without the 17-hydroxyl had no effect. Recently, Langecker (78) has given intramuscular injections of 17α-hydroxyprogesterone to normal male human beings. Urinary excretion of pregnane-3,17,20-triol and pregnane-3,17-diol-20-one was increased, but the output of 17-ketosteroids and the pregnane-3,20-diols remained unchanged.

Following the oral administration of hydrocortisone to a patient with Hodgkin's disease, Burstein et al. (79) isolated increased amounts of the following C_{19} steroids: etiocholane-3α,11β-diol-17-one, 9,11-etiocholene-3α-ol-17-one and etiocholane-3α-ol-11,17-dione. Riegel et al. (80) and Gallagher et al. (81) showed that mice injected intraperitoneally with progesterone-21-C^{14} were able to remove part of the radioactive carbon and excrete it in the expired air as $C^{14}O_2$. As demonstrated by Axelrod and Miller (82), these conversions could all be due to degradative reactions in the liver and not related to biosynthesis of androgens in the testis.

It was not until this year that Slaunwhite and Samuels (83) obtained direct evidence that testis tissue *in vitro* is able to convert progesterone to testosterone and androstenedione. The likely involvement of progesterone had previously been indicated by the work of Samuels and co-workers (84), even though progesterone has not yet been isolated from testis tissue. Their experiments showed that all normal endocrine tissues which produce non-benzenoid steroid hormones contain an enzyme which will oxidize certain 5,6-dehydro steroids with a 3β-ol group to the corresponding α,β unsaturated 3-ketone, the latter structure being characteristic of the more biologically active 19-carbon and 21-carbon steroids. Thus, there was shown to be present in testis tissue an enzyme system which could synthesize progesterone, given the proper substrate. This enzyme system, as constituted *in vitro*, involved diphosphopyridine nucleotide (DPN) as electron acceptor and has been termed a 3β-ol dehydrogenase.

In their initial experiments (84) it was further found that testis tissue from cryptorchid rats treated with chorionic gonadotropin was able to convert pregnenolone to progesterone. In later work (85), it was found that testis tissue from normal untreated rats also contained this enzyme system, and that tissue from hypophysectomized rats treated with HCG was about three times more active. No data were given for intact rats treated with HCG, however.

A recent study (86) on the relation of 3β-ol dehydrogenase activity to androgen production in interstitial-cell tumors of mice has shown that androgenic activity of the tumor transplant was always absent when there was no measurable 3β-ol dehydrogenase activity.

It would seem, therefore, that as in the case of the adrenal cortex (71)

the sequence of hormone production involves the conversion of some precursor, perhaps cholesterol or a congener, to pregnenolone. It will be recalled that pregnenolone has indeed been isolated from swine testis tissue (87). Pregnenolone is then converted to progesterone, and a variation in the subsequent steps produced the endocrine products particular to a given gland.

Details of the Work of Slaunwhite and Samuels (83).

The research of this thesis was a direct continuation of the experimentation of the above two authors. Because of this and because the work is not yet in print, it is pertinent to consider the material in some detail. Furthermore, some of the findings not prepared for publication had a definite influence on the approach to the problems of this thesis.

All the results of their experiments were obtained by the incubation of testis tissue in a serum-buffer medium in the presence of steroid substrate, either progesterone-4-C^{14} or progesterone-21-C^{14}. Nicotinamide, diphosphopyridine nucleotide, adenosine triphosphate, and fumaric acid were also provided in the medium. Two types of tissue preparations were used: one was a cell dispersion prepared by violently shaking minced, decapsulated testes; the other was a homogenate. In some cases, the testes were from hypophysectomized rats treated with HCG; in others normal immature rats had been treated with HCG. Treatment was for 10-14 days and each rat received a total of 1400 to 2000 international units (I.U.) of the gonadotropin.

In one experiment, it was found that two testes (1.5 gm. tissue) in the form of a dispersion were able to convert 50 per cent of the added progesterone-21-C^{14} (1 μmole) to C_{19} compounds in 3 hours of incubation. The aqueous incubation mixture was made alkaline and extracted with ethyl acetate.

The radioactivity was then measured in the ethyl acetate extract and the alkaline water. Control flasks had been run to determine the amount of radioactivity due to progesterone-21-C^{14} which remained in the aqueous phase as a result of incomplete extraction; this was found to be 5 per cent. Of the flasks incubated with testis tissue, the amount of radioactivity in the aqueous phase above the control value was considered to reside in the "fragment" formed as a result of the enzymatic scission of carbons no. 20 and 21 during the formation of androgens (C_{19} compounds) from the C_{21} substrate.

Identification of this radioactive scission product was attempted. The major portion of the radioactivity in the aqueous phase after extraction was found to be steam-distillable from acid solution but not from alkaline solution. About 6 per cent of the steam-distillable radioactivity was found to be formic acid based on conversion to carbon dioxide by reaction with potassium permanganate. It was tentatively considered that the major portion of the steam-distillable acid was acetic acid.

Steroids identified as products of the incubation of progesterone-4-C^{14} with testis tissue were 17α-hydroxyprogesterone (17-OH-P), 4-androstene-3,17-dione, and testosterone. Characterization was by paper chromatography, acetylation with pyridine and acetic anhydride at room temperature, and recrystallization to constant specific activity in the presence of added carrier. It was observed that when testis homogenates were used, no formation of testosterone took place even though there was formation of both 17-OH-P and androstenedione and of an unidentified compound of less polarity than progesterone. Total conversion was decreased by about half, however. Because of their importance, the data are reproduced here in Table I.

Table I †

Proportion of Radioactivity in Various Peaks Associated with Specific Compounds as Per Cent of the Summed Activities of the Peaks (Experiment 4)

Flask No.	3	6
Testis Preparation	Dispersion	Homogenate
Position C^{14}	4-C^{14}	4-C^{14}
Areas due to:	%	%
17α-Hydroxyprogesterone	3.6	7.0
4-Androstene-3,17-dione	21.2	6.7
Progesterone	45.3	73.7
Testosterone acetate*	29.9	0
Low Polarity Unknown		12.6

† From Slaunwhite, W. R. Jr., and Samuels, L. T. J. Biol. Chem. (In press).

* Radioactivity moved from 17α-hydroxyprogesterone-testosterone spot to testosterone acetate spot on acetylation.

It is important to note that the above work has shown that rat testis, in common with the adrenal cortex, not only has a 3β-ol dehydrogenating enzyme system, but also a 17-hydroxylating one. On the basis of the information obtained from their work, Slaunwhite and Samuels postulated the following sequence of events in the production of androgens by testis tissue.

It was the problem of this thesis, then, to investigate more fully the above conversion with special emphasis on the factors and steps involved in

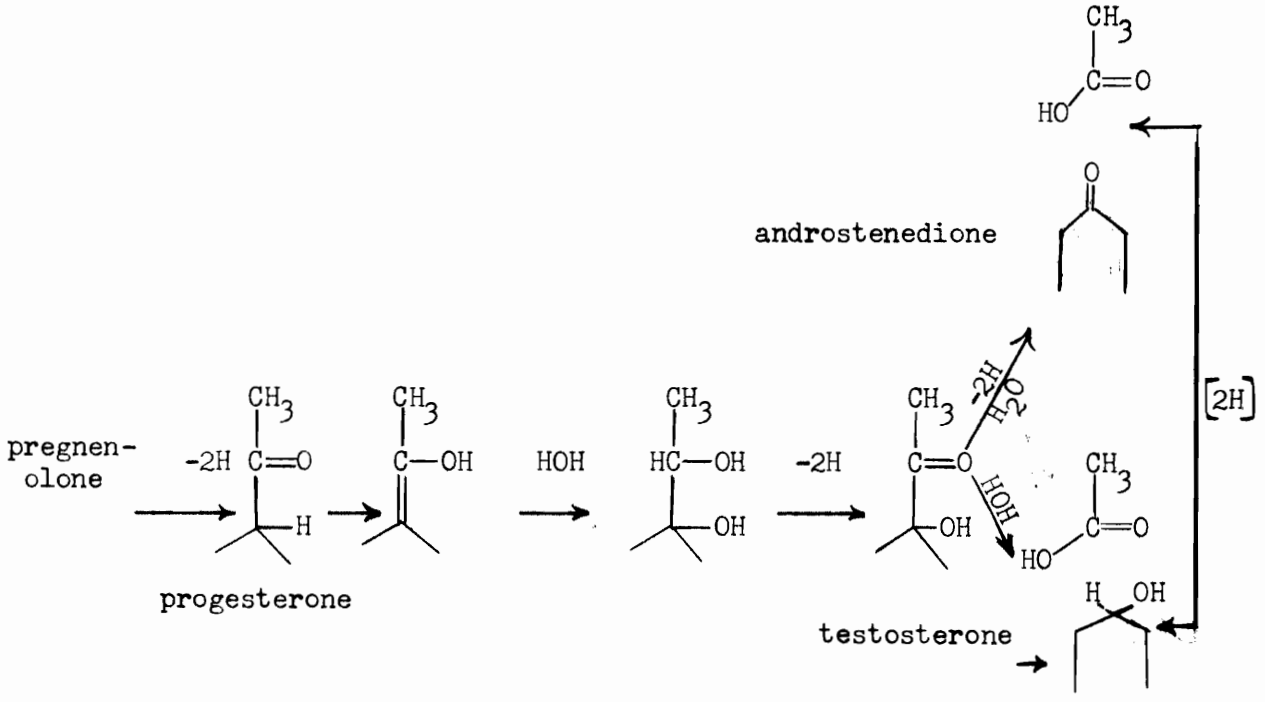


Figure 1. Postulated Sequence in Conversion of Pregnenolone to Testosterone and Androstenedione by Rat Testis Tissue.

the specific reaction whereby the C_{21} compound, presumably 17α-hydroxy-progesterone, is converted to a C_{19} androgen, either androstenedione or testosterone or both.

Summary of the Historical Review.

Testosterone appears to be a testicular hormone in several species. In man and dog, however, two androgens - testosterone and androstenedione - have been identified as hormonal secretions of the gonad, and the rat testis has been shown to be able to synthesize these compounds in vitro. There is almost unanimous agreement that the cellular locus of synthesis of these androgens is the interstitial, or Leydig, cell, which appears to be primarily under control of a glyco-protein of the anterior pituitary gland, called interstitial cell stimulating hormone (or ICSH). Experimentally, high

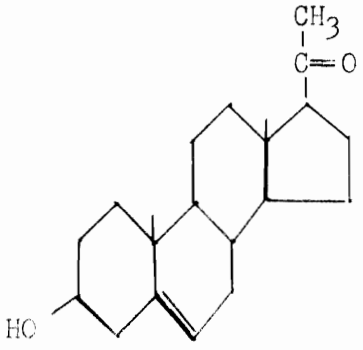
activity may be induced by the injections of human chorionic gonadotropin (HCG).

The exact biochemical role of the gonadotropins has not been determined. Studies in the steps of biosynthesis of the testicular hormones may allow one to determine at what point these protein hormones have their tropic influence, how androgen production is controlled, and how various regulatory mechanisms are affected.

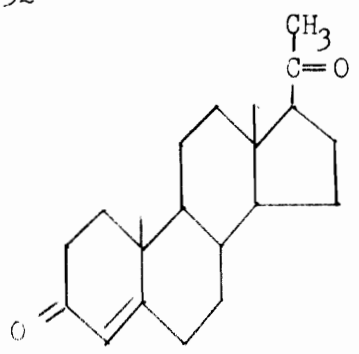
To date, it has been shown that testosterone and androstenedione can be synthesized *in vivo* and *in vitro* from acetate and that chorionic gonadotropin is effective under both conditions. Nothing else fundamental is known about *in vivo* conversions. The first compound from which a sequence of reactions leading to C_{19} compounds has been demonstrated *in vitro* is 5-pregnen-3β-ol-3-one.

Pregnenolone is converted to progesterone, which seemingly by way of 17α-hydroxyprogesterone can proceed either to testosterone or to androstenedione. Whether one androgen is formed first and is converted to the other or whether each comes from progesterone by a separate pathway is not known. Testis cell dispersions and homogenates have not yielded the same products from progesterone: both androstenedione and testosterone appear in the former case, but only androstenedione in the latter.

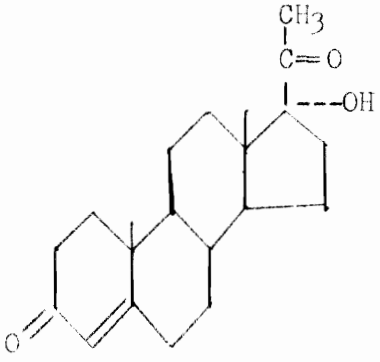
Nothing is known about cofactor requirements for the conversion of progesterone, and there is no information regarding the cellular components which are responsible for the enzymatic conversions.



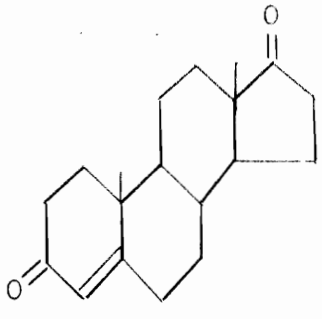
Pregnenolone



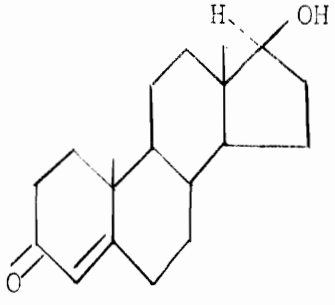
Progesterone



17α-Hydroxyprogesterone



Androstenedione



Testosterone

Formulas of Steroids

IV. EXPERIMENTAL

A. Materials and Methods

In this section, only those materials and methods in general use are presented. Deviations from general procedure and additions thereto are noted in connection with the individual experiments.

1. Preparation of Animals and Tissue.

Male Sprague-Dawley rats were used in all experiments. Prior to sacrifice, they were given intramuscular injections of 100 I.U. human chorionic gonadotropin (HCG)[1] per day for about two weeks. In the earlier experiments, hypophysectomized rats were used; later, immature rats were preferred. The rats were sacrificed by quick decapitation; the testes were removed, decapsulated, and minced on a glass plate with a razor blade. In some cases a single pair of testes was added to the incubation flask. In most instances, however, the minced testes were combined in a convenient volume of medium and mixed well by shaking violently; in this way fairly uniform aliquots could be added by pipette to a series of flasks. Each individual flask was then shaken violently just prior to incubation. This testis preparation is referred to as a dispersion.

Some incubations were carried out by treating the testis dispersion for about a minute with a "POLYTRON Hochfrequenzdispergier und Homogeniseirgerat".[2]

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[1] Sincere thanks is extended to Ayerst Laboratories, Inc., New York City, and to Mr. R. Rivers of that company for generous supplies of A.P.L.

[2] Licensed by Prof. P. Willems. Obtained from Werkstatten fur Prazisionsmechanik und Apparatebau. MAX WULLIMAN, Selzach, Schweiz.

This instrument rapidly destroys cell boundary structures with no foaming. This testis preparation is referred to as a homogenate.

Methods of cell fractionation will be discussed in connection with the individual experiments.

2. Cofactors and Substrates.

All cofactors and substrates were obtained from commercial sources; no assays or analyses were made except that steroid substrates were checked by paper chromatography in a suitable system. The 4-androstene-3,17-dione (hereafter referred to as "androstenedione") and testosterone used were from laboratory stock solutions, the original source of which was not known with certainty. 17α-Hydroxyprogesterone was obtained from Mann Research Laboratories, New York 6, New York. Progesterone-21-C^{14} was purchased from Charles E. Frosst and Company, Montreal, Canada. The following cofactors were obtained as listed:

Adenosine triphosphate, disodium salt (ATP). Pabst, Lots 101 and 131, 95% pure.

Diphosphopyridine nucleotide (DPN). Pabst Lot 310, 92% pure.

Triphosphopyridine nucleotide (TPN). Sigma Lot 124-85, 78% pure.

Triphosphopyridine nucleotide (TPN). Sigma Lot 45-633, 97-99% pure.

Reduced DPN (DPNH). Sigma Lot 82-111, purity unknown.

Reduced TPN (TPNH). Sigma Lot 85-626, 80% pure.

Flavin adenine dinucleotide (FAD), Sigma Lot 94-623, 49% pure.[1]

Coenzyme A. Sigma Lot C25-91, 70-75% pure.

Thioctic acid. Lederle Lot 7-8019.[2]

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[1] Generously supplied by Sigma Chemical Company.

[2] Thioctic acid was obtained by the combined courtesies of Lederle Laboratories and Dr. H. W. Davenport.

(The above cofactors were kept cold, dark, and dry).

Fumaric Acid. Fischer, pure.

Sodium Fumarate. Chemical Commerce, Newark 5, New Jersey.

Nicotinamide. Merck, U.S.P.

3. Methods of Incubation.

In nearly all cases, incubations were carried out in a glass-stoppered (S) 125 ml. Erlenmeyer flask using 25 or 30 ml. of a 1:1 mixture of Krebs-Ringer bicarbonate buffer and bovine serum at a pH of 7.2-7.3. The composition of the K-R buffer is given in Table II. Unless otherwise noted, cofactor additions were made to give a concentration of 0.4 millimoles (mM) per liter; thus, for each 25 ml. incubation medium, there were 10 micromoles (μM) of cofactor present.

Table II

Composition of Krebs-Ringer Buffer

Salt	Grams per 500 ml. solution
NaCl	90
$NaHCO_3$	27.3
KCl	4.6
$MgSO_4 \cdot 7H_2O$	3.8
$CaCl_2$	3.7
KH_2PO_4	2.1

5 ml. of each of the above solutions was taken; to the mixture was added 100 ml. water. The mixture was gassed for 10 minutes with 95% O_2/5% CO_2.

ATP and fumarate were routinely included as in the studies of Hayano and Dorfman (88) on the 11-hydroxylase system of the adrenal cortex. A discussion of these factors as requirements for the particular enzyme systems being studied here occurs in connection with the individual experiment concerned.

Nicotinamide (0.04 molar) was used when either DPN or TPN was included in the incubation medium. When the latter were not used, neither was nicotinamide. Nicotinamide was provided to inhibit the hydrolysis of DPN and TPN. The presence of a DPN-ase in animal tissues was first shown by Mann and Quastel (89) in 1941, and they also showed that nicotinamide was capable of inhibiting the process. Shortly thereafter, Handler and Klein (90) reported that TPN was also attacked, and that the product of the hydrolysis was nicotinamide. Thus, the enzyme is a nucleosidase. In experiments with broken cell suspensions of rabbit brain, Handler and Klein showed that 0.04 molar nicotinamide gave moderately good protection against nucleosidase activity; 0.08 molar nicotinamide was somewhat better but not proportionately so. There was no inhibition of DPN or TPN activity by the high levels of nicotinamide. It is interesting to note that nucleosidase has been reported not to attack DPNH and TPNH (91).

Explanation of the use of DPN, TPN, their reduced forms, and of the use of the other cofactors will be given in connection with the individual experiments.

Steroid substrates were provided by adding the required amount of alcoholic solution to the flasks before any other additions had been made. The alcohol was then evaporated, and in some cases the steroid was redissolved in 0.2 ml. alcohol. In others, the bovine serum was used as the effective solvent.

Routinely, before any cofactors were added to the stock solution of freshly prepared serum-buffer the mixture was thoroughly aerated by bubbling through a 19:1 mixture of O_2-CO_2, both to adjust the pH of the mixture and to saturate it with oxygen. After addition of the incubation ingredients, each flask was again gassed for 3 minutes before final stoppering. When a nitrogen atmosphere was to be provided, each individual flask was gassed for 7-8 minutes with a 19:1 mixture of N_2-CO_2, with occasional swirling of the contents.

Each flask was then tightly stoppered; usually, a wax was used to effect a tight seal, although results show that this was not accomplished in all cases. The flasks were then placed in a water bath at 34° C. and rotated end over end for 3 hours. At the end of this time, they were removed and immediately frozen to await later extraction.

4. Methods of Extraction.

After thawing, the incubation medium was adjusted to pH 8-9 with KOH and transferred to a 100 ml. round-bottom centrifuge tube, using one 5 ml. water wash, and two 15 ml. ether washes. Thorough mixing was then accomplished with a glass propeller type stirrer attached to an electric motor. After centrifuging to effect a sharp separation of the two phases, the ether phase was carefully drawn off by means of a "serum lifter". Extraction of the aqueous phase was repeated twice more with 30 ml. ether. The combined ether extracts were adjusted to 75 ml. and back-extracted with 5 ml. water. After centrifuging, the water layer was carefully removed with a long hypodermic needle attached to a syringe and added to the original aqueous fraction.

In the cases where a non-radioactive substrate such as 17α-hydroxy-

progesterone had been used, the water fractions were discarded, and the ether fractions were submitted to paper chromatography as will be explained. When radioactive substrates had been used, the aqueous and ether fractions were handled as explained in the next section.

5. Measurement of Radioactivity.

a. Aqueous fractions.

The alkaline aqueous fractions after ether extraction were measured for total volume (usually in the range of 40 to 50 ml.), and two aliquots of 200 lambda each were plated on round aluminum discs 25 mm. in diameter. Counting of the radioactivity was done in windowless gas flow counters. Corrections for self-absorption were made using a $BaCO_3$ self-absorption curve. The validity of this correction curve was checked by plating equal aliquots of the same aqueous phase over different areas and by plating different aliquots over the same area. Total radioactivity of the water fraction was then calculated.

b. Ether fractions.

The ether extracts[1] were dried down completely under a stream of nitrogen, the tubes being placed in a water bath at 40° C. The residue in each tube was redissolved in 20.0 ml. ethanol, and two aliquots of 100 lambda each were taken for plating. Counting of the radioactivity was done as above. Corrections for self-absorption were made using a wax self-absorption curve, although usually the weight of the material on each plate was negligible. Total radioactivity of the ether fraction was then calculated.

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[1] One drop glacial HOAc added to each tube before drying down.

c. Derivation of results from the radioactivity data.

In the previous experiments of Slaunwhite and Samuels (83), it had been shown that when progesterone-4-C^{14} was used as the substrate, about 5 per cent of the radioactivity remained in the aqueous fraction after ether extraction, no matter to what extent the progesterone had been converted to other compounds. In those incubations in which progesterone-21-C^{14} was used, there was considerable variation in the ratio of radioactivity in the aqueous and ether phases. It was shown that the percentage of radioactivity in the aqueous phase was a general measure of the enzymatic activity of the testis tissue used in the incubation. This was so because in conversion of the progesterone-21-C^{14} to C_{19} steroids, the side chain carrying the radioactive label was removed and converted to a water-soluble acid. The radioactivity of the ether phase as measured was, therefore, assumed to be 95 per cent of the actual ether-soluble radioactivity and the measured values were corrected accordingly, the correction being added to the values for the ether-soluble fraction and subtracted from those of the aqueous phase. This type of calculation is illustrated in Table III.

This type of calculation, then, was used as an indication of the extent of conversion of the substrate progesterone to C_{19} compounds and as a measure of the amount of enzymatic activity. Information concerning identity of the C_{19} compounds and concerning C_{21} intermediates was not obtained until chromatography had been carried out.

6. Paper Chromatography.

The ether phase material had been dissolved in alcohol for plating of aliquots. Afterwards, the alcohol was evaporated under a stream of nitrogen,

Table III

Calculation of Radioactivity of Aqueous and Ether Phases as a Measure of Conversion of Progesterone-21-C^{14} to C_{19} Compounds by Testis Tissue.

Flask	Radioactivity in Counts per Minute				
	Aqueous (alkaline)	Ether	Total	% Aqueous	% Conversion
Control	19,450	369,000	389,000	5	--
A	216,000	176,000	392,000	55	--
A Correc.	207,000	185,000	"	53	53
B	64,000	328,400	392,900	16	--
B Correc.	46,400	346,000	"	12	12

and ethyl acetate was introduced as the solvent. Some material always failed to dissolve; hence, filtration through a medium glass sintered funnel was carried out into a 40 ml. conical centrifuge tube followed by proper washings. The solvent was then evaporated and the residue carefully concentrated into the conical tip in preparation for application to a paper chromatogram. Since the mixture always contained considerable fat and other materials less polar than the steroids to be investigated, it was necessary to get rid of this less polar material so that subsequent resolution of the steroids themselves could be accomplished in other chromatographic systems. This first purification was done by using the reversed phase system of Martin and Bush (92). The development and use of this system will be described in some detail as well as the results obtained thereby.

a. Development and use of the reversed phase system.

This particular system was developed originally in order to assist in the study of cholesterol transformations in the rat and dog adrenal. Methods previously available for the separation on paper of the less polar steroids were considered unsatisfactory for one or more reasons. A Quilon-impregnated paper as suggested by Kritchevsky and Kirk (94) is opaque to ultraviolet light and the use of the Haines Ultraviolet Scanner (95) is thus precluded. Furthermore, the Quilon interferes in later manipulations.

The method of Neher and Wettstein (96) uses phenylcellosolve as the impregnating phase which must later be removed for any subsequent operations; this removal apparently requires heating in a 90° C. oven for half an hour or drying for lengthy periods at room temperature. This can be very undesirable as shown on p. 54 . Furthermore, such a system does not have the advantages of having the non-polar phase as the stationary one. Thus, the system has a relatively low capacity because a large amount of "fat" tends to carry the more polar steroids along as it moves to the front. In a reversed phase system, the very non-polar materials remain at the origin, allowing a more satisfactory resolution in the off-origin part of the paper.

The Zaffaroni battery of systems (11) is not adequate for the chromatography of cholesterol, and furthermore, some difficulty is encountered in removing either propylene glycol or formamide which often interfere in later manipulations. For example, residual formamide on the paper has been found to interfere with certain steroid color reagents such as phosphomolybdic acid in the detection of alcohols and the Zimmermann reagent in the detection of ketones.

As a result of the above considerations, the reversed phase system of Mills and Werner (93) was adapted for steroids. These English workers used a high-boiling hydrocarbon called "odorless kerosene" as the stationary phase

and aqueous iso-propanol as the developing phase to separate various resins. Their system was first tried here for the resolution of ether-soluble materials obtained from rat and dog adrenal glands and was considered not to be satisfactory. Finally, 60 per cent aqueous n-propanol was found to be quite effective as the developing solvent. The details of the procedure follow.

Circular chromatography tanks 12 inches in diameter and 24 inches high have been used. The inside wall was lined with filter paper except for a gap to allow one to observe the papers as chromatography progresses; this paper dipped into a 60 per cent aqueous n-propanol solution which covered the bottom of the tank to a depth of about 2 inches. In the center was placed a 600 ml. beaker fitted with a rolled filter paper which extended nearly to the top of the tank; this beaker was filled with odorless kerosene (O.K.).[1] Tanks so prepared have been used for at least 8 months without need for reconstitution.

To prepare the chromatograms, Whatman filter paper No. 2 was impregnated with O.K. by dipping the paper in 12 per cent (v/v) O.K. in Skelly-Solve C, or some other suitable volatile solvent. This dipping has been most easily accomplished by rolling the paper and placing it in a wire basket which can be lowered into a large glass cylinder containing the impregnating solution. After the paper was drained momentarily, it was evenly blotted between two cardboards with layers of filter paper attached. The paper was then supported at both ends on a rack to keep it from touching any large surfaces while the

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[1] n-Propanol has been obtained from Matheson, Coleman and Bell and was used without further purification. Odorless kerosene has been obtained locally from Wasatch Chemical Company. It is a Standard Oil Company product called "Base Oil C". Boiling range is given as 180-325° C. It has been used without further purification.

material to be chromatographed was being applied.[1]

Usually a preparative type of chromatogram was run to obtain resolution of as much material as possible. Thus, the paper was cut to be 175 mm. wide. The origin line was placed at least 9 or 10 cm. below the line at which the paper was suspended by the glass rod in the tank.

As previously mentioned, the extract material to be chromatographed had been concentrated into the bottom of a conical tube. It was redissolved in about 200 lambda of ethyl acetate and applied by micropipette in as thin a line as possible at the origin to within 1 cm. of each side of the paper. Two 100 lambda washes were applied directly on top of the original application. Since the impregnating phase was displaced on the paper during this operation, a 10-12 hour equilibration in the tank was required. This allowed redistribution of the O.K. and a spreading out of the applied material. When smaller amounts of material were applied and the original displacement was less, the equilibration time was decreased to 5 or 6 hours.

Because of its availability in this Department, a constant temperature room set for 37 $\pm$ 1° C. has been used. However, variations of about 5 degrees have seemed not to affect the development of the chromatograms. It was found also that the chromatograms could be equilibrated and developed equally well at a room temperature of about 22°. Thus, to use this system one does not need a chamber of elevated temperature, and some temperature variation can be tolerated.

After the papers had equilibrated, the descending solvent, 60 per cent

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[1] The rack was easily made from a set of Tinker Toys.

aqueous n-propanol saturated with O.K., was run in from the bottom of a separatory funnel through two 1 cm. holes drilled in the glass plate tops to fit just over the tops of the troughs in the tank; the holes were stoppered with corks. Optimal resolution times depend on the results one desires. Generally, for relatively pure compounds a 10 to 12 hour period is sufficient and for crude extracts, 12 to 15 hours. However, shorter periods may give the separation required. The solvent front was always visible by back-light illumination when the paper was in the tank and could be easily seen when the paper was first removed from the tank. Furthermore, a line marking the front was visible when the dry paper was viewed in the Haines Ultraviolet Scanner. Thus, the solvent front could be marked exactly and R_f's were quite accurately calculated for the individual compounds.

Fortunately, after removing the paper from the tank a short drying period of about 15 to 20 minutes at room temperature was sufficient for further operations such as viewing in the UV scanner, spraying with various color reagents, elution and immediate application to other resolving systems, or elution, plating, and counting for radioactivity. If any residual solvent has ever been present, it has never interfered with any of the manipulations mentioned.

For application of color reagents, a strip 4 mm. wide was cut lengthwise from the center of the paper. Spraying with an 8 per cent alcoholic solution of phosphomolybdic acid (94) has been very useful for the detection of cholesterol, its esters, and other similar compounds. Uniform color development has been achieved by placing the sprayed strip in the hot room

(37° C.) for about ten minutes. By this type of heating, one can get optimal color development with a minimum of background color from the paper.

A facsimile of a reversed phase chromatogram, drawn to a 1:4 scale, is shown in Fig. 2. An ether extract of rat adrenal tissue had been applied at the origin and developed as described. The center 4 mm. strip was sprayed with phosphomolybdic acid to delineate the areas shown by the dotted lines. The prefrontal area also was determined by viewing in the UV scanner since UV absorbing material was always present in this region.

The practice of Rosenkranz (97) has also been folowed by using antimony trichloride in nitrobenzene. It was more sensitive for cholesterol detection than phosphomolybdic acid but not as convenient to use. The color obtained with $SbCl_3$ and cholesterol has been consistently a pink or red in white light and a pink fluorescence in UV light rather than a blue-violet and light blue, respectively, which Rosenkranz reported.

A reagent apparently not previously applied to steroids, phosphotungstic acid, has been used with considerable success. A 15 per cent alcoholic solution has been used as a spray to detect compounds such as cholesterol and pregnenolone. This reagent was not as sensitive as phosphomolybdic acid but it was much more specific as will be noted later.

After the various areas had been located by using the UV scanner or by spraying the center strip, the chromatogram was cut so that the material could be eluted from the paper. In general, the large areas were eluted in a Soxhlet apparatus. Ethanol was used to elute the prefrontal area, ethyl acetate was employed for the cholesterol region, and ethyl acetate or ether for the origin. It should be mentioned that in one experiment where

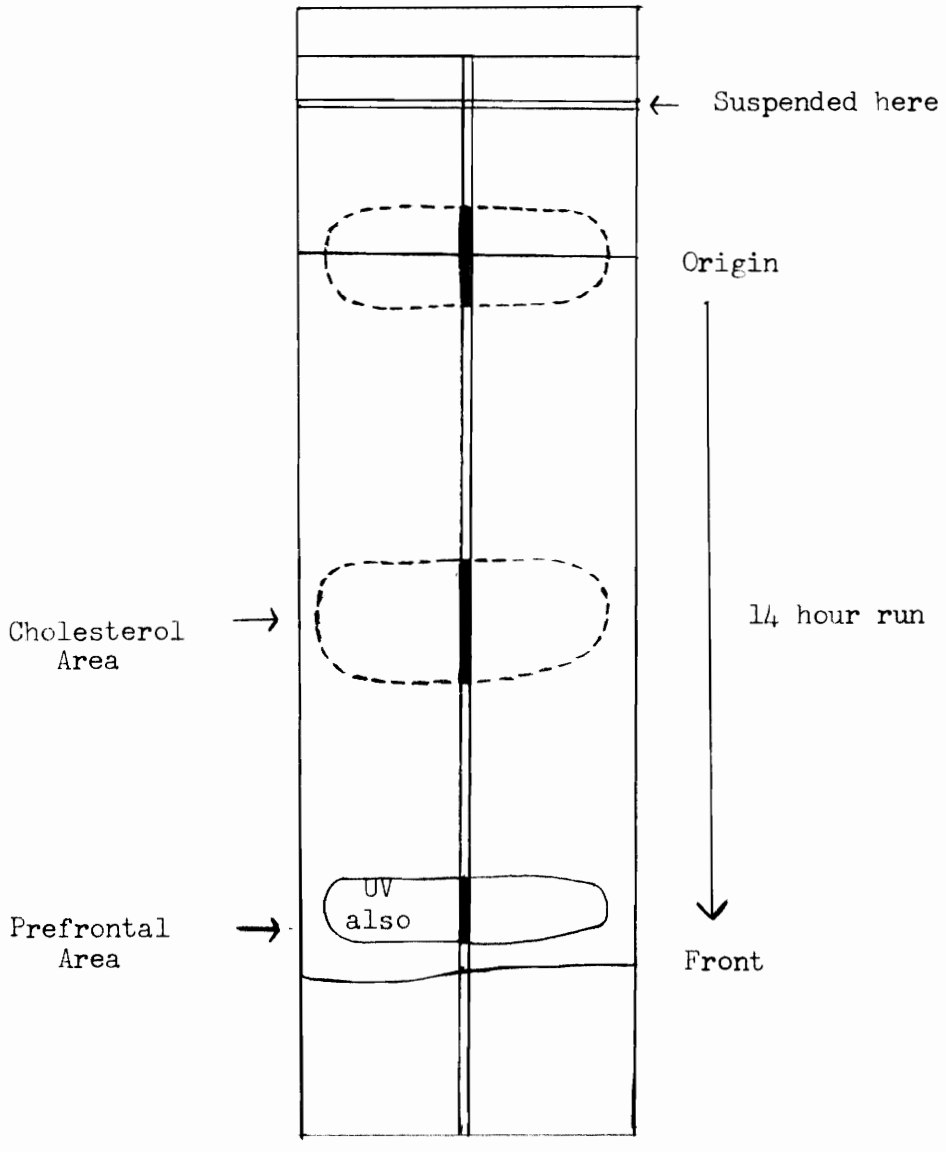

Figure 2. Facsimile of Reversed Phase System Chromatogram.

"Odorless Kerosene" - Stationary Phase
60 per cent Aqueous n-Propanol - Developing Solvent

Facsimile of developed chromatogram. Ether extract of rat adrenal tissue has been applied at origin. Center 4 mm. strip has been sprayed with phosphomolybdic acid to delineate the areas as shown by dotted lines.

it was desired to elute natural adrenal cholesterol esters from the origin, ligroin was used instead of ether. It was subsequently found that ligroin did not elute the compounds in the three-hour period of refluxing and that a solvent such as ether or ethyl acetate was necessary.

When the material in the prefrontal region was present in a rather thin compact area, then elution was carried out at room temperature in an elution chamber fitted with a flat-bottomed stainless steel trough (2 x 8 x 68 cm.). A special metal frame held a filter paper which dipped into ethanol in the trough; the solvent was thus delivered slowly by capillarity up and over the top of the trough to a part of the filter paper fitted vertically between the metal frame and a glass plate held in place by movable clamps. Thus, the top half centimeter or so of each individual chromatogram section could be placed between the filter paper and the glass plate; when the latter was again clamped in place there was good contact between the solvent-delivering filter paper and the pendent strip from the chromatogram. The lower end of the chromatogram paper was bevelled so that the eluent would flow down the entire paper strip, then concentrate at the point in the center to drop into a collecting flask. To prevent evaporation of eluent, the cabinet was kept saturated with alcohol by having four large filter paper pads line the sides of the chamber and dip into a tray in the bottom filled with alcohol.[1]

In general, a flow of about 5 ml. of eluent was sufficient to elute 50 µg. amounts of steroids. Micromolar amounts required much more time and

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[1] This system for cold elution of several small papers at a time was designed by Dr. Boyd W. Harding.

solvent.

Another method of elution can be used when it is desired to collect total off-origin material. The chromatogram is cut to be about 30 cm. long with a sharp bevel leading to a point in the center of the lower edge. A cylinder with funnel is placed directly in the chromatography tank beneath the pointed end of the paper; in fact, the tip of the paper may touch the inside of the funnel to drain off the drops of developing solvent as they collect. Thus, a chromatographic separation and elution may be accomplished at the same time. If undesirable material follows the prefrontal material too closely, this method cannot be used successfully.

b. Results obtained by using the reversed phase system.

Results with individual compounds: A list of compounds and their R_f values in the reversed phase system is given in Table IV. These values were obtained by placing about 50-60 μg. of the known compound at the origin, developing as previously described, then locating the compounds by spraying with phosphomolybdic acid or by viewing in the UV scanner. Special notice should be taken of the compounds which run in the prefrontal region. One can make the general statement that steroids having a "polarity" equal to or greater than 7-ketocholesterol appear in this area.

It is also evident from the table that at least two other compounds have the same R_f as cholesterol at the end of a 12 hour development. Some separation of these compounds might be accomplished by extending the developing period to 20 or 24 hours, but this was not attempted.

Commercial "pure" cholesterol when chromatographed by this system gave two phosphomolybdic acid-positive spots, one at the prefront and one at R_f

Table IV

R_f Values for Reversed Phase System on Paper

Stationary phase: odorless kerosene.
Descending phase: 60 per cent aqueous n-propanol.

Compound (50-60 μg.)	R_f	Phosphomolybdic acid*	U.V.†
Cholesteryl palmitate	0.00	+++	0
" acetate	.14	+++	0
4-Cholesten-3-one	.32	0	+
3α-Cholestanol	.40	+	0
3β- "	.46	++	0
Cholesterol	.55	+++	0
7-Cholesten-3β-ol	.55	++	0
7,9-Cholestadien-3β-ol	.55	++	0
4-Cholestene-3,6-dione	.65	0	+
Pregnenolone-3-methyl ether	.75	++	0
Testosterone-17-methyl ether	0.85	0	+
7-Ketocholesterol	.87	0	+
4-Cholesten-6β-ol-3-one	.87	0	+
4-Cholesten-3β-ol-6-one	.87	++	+
Progesterone	.87	0	+
Testosterone acetate	.87	+	+
Androstenedione	.87	0	+
Testosterone	.87	+	+
17α-Hydroxyprogesterone	.87	0	+
17α-Methyltestosterone	.87	+++	+
Corticosterone	.87	0	+
Cortisol	.87	0	+

* Paper was sprayed with 8 per cent alcoholic phosphomolybdic acid solution and heated at 37° C. for 10-15 minutes; relative intensities of dark blue color indicated by +'s.

† Paper was viewed in the Haines Ultra Violet Scanner.

0.55. When the impure cholesterol was purified via the 5,6-dibromide (84)[1] and rechromatographed, it gave only one spot at an R_f 0.55. The reversed phase system was also used to check the purity of cholesterol-4-C^{14}.[1] The crystalline product first obtained exhibited two radioactive peaks, one at the prefront and one at the cholesterol area. Upon careful recrystallization, the more polar substance was eliminated. This is shown in Fig. 3. The dotted line illustrates the radioactive impurity. The solid line illustrates the chromatographically pure cholesterol-4-C^{14}. The black block illustrates the phosphomolybdic positive area, showing the close coincidence of the color and the radioactivity.

Phosphomolybdic acid test: As phosphomolybdic acid was used here, the Δ^4-3-keto group was not reactive. Testosterone was only slightly positive. The saturated 3-ols were not as positive as those with a double bond; 3β-cholestanol was more reactive than the epimer. The tertiary alcohol, 17α-methyltestosterone was surprisingly quite positive. The α-ketol group of corticosteroids was not reactive.

Special attention is called to 7-ketocholesterol and to 4-cholesten-6β-ol-3-one. These compounds gave no color whatsoever with phosphomolybdic acid under the conditions described. It has been suggested that in these compounds a dehydration first occurs which prevents the usual oxidation-reduction reaction.[2]

Phosphotungstic acid test: When cholesterol on paper was sprayed with a 15 per cent alcoholic solution of phosphotungstic acid and placed in

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[1] Prepared by Dr. George I. Fujimoto.

[2] Dr. George I. Fujimoto.

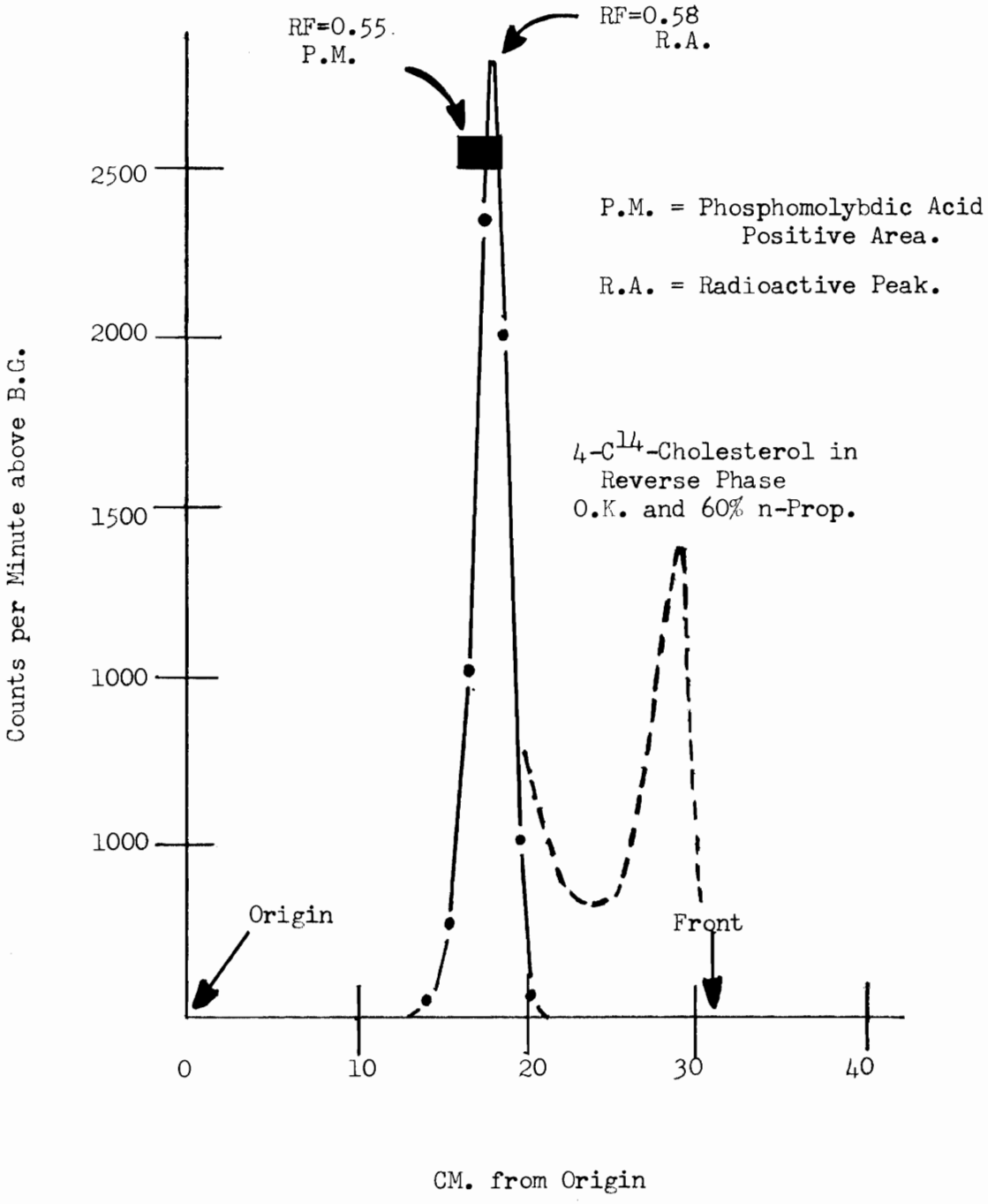

Figure 3. Distribution of Radioactivity by Chromatography of 4-C^{14}-Cholesterol.

the warm room at 37° C. for color development, the area first became yellow, then gradually turned pink; the latter color became more intense with time. In general, this reaction was given by 5,6-dehydro-3-ol-steroids. As seen in Table V, other groups may interfere, and other colors may be obtained.

It was found that the compounds which gave a definite pink color were those which have a double bond β-γ to a 3β-hydroxyl group. (In this series, no 3α-ols were available). As with phosphomolybdic acid (PMA), other groups present can interfere; it is interesting to note that 4-cholesten-3β-ol-6-one, which was positive to PMA, produced the yellow color with phosphotungstic acid but progression to pink did not occur. The results with the Δ^4 compounds suggest that this reagent can distinguish hydroxyl groups with α,β unsaturation from those with β,γ unsaturation, and further, that position 3 epimers may be distinguished. More known compounds should be tested before a generalization is made, however.

Results with tissue extracts.

Adrenal tissue: The reversed phase system has a rather high capacity for holding "fat" at the origin and for preventing compounds such as cholesterol from entering the prefrontal region. Poor chromatograms can easily result, however, from having too much polar material present in the extract. Excellent initial separations into origin material, "cholesterol", and prefrontal compounds have been accomplished by applying as much as 35-40 mg. of EtOAc-soluble material, derived from 500 mg. of rat adrenals, on a paper 175 mm. wide.

Mention was made earlier of the general desirability of freeing the paper of the chromatography solvents and of eluting the compounds as quickly as

Table V

Reactions of Steroids on Paper After Spraying With Phosphotungstic Acid

Steroid	Color Development
5-Cholestene	Slight discoloration
7-Cholesten-3β-ol	" "
7,9-Cholestadiene-3β-ol	" "
Cholestan-3α-ol	" " (light tan)
Cholestan-3β-ol	" " "
Cholestan-3-one	No color
Cholest-4-en-3-one	"
3β-Methoxyandrostan-17-one	"
11-Desoxycorticosterone	"
4-Cholesten-6β-ol-3-one	Very light tan
4-Cholesten-3β-ol-6-one	Light yellow
3β-Methoxyandrostan-17-one	Light yellow
Sitosterol acetate	Positive: pink
Cholesterol	" "
Cholesteryl acetate	" "
Cholesteryl palmitate	" "
7-Ketocholesterol	Very slightly positive (pink)
7-Ketocholesteryl acetate	Positive: pink
5-Pregnen-3β-ol-20-one	" "
17-Hydroxypregnenolone	" "
5,6-Dehydroepiandrosterone	" "
5-Androstene-3β,17β-diol	" "
17α-Methyl-17β-hydroxy-5-androsten-3β-ol	Pink or orange-pink
17α-Methyl-17β-hydroxy-4-androsten-3β-ol	Lemon yellow to orange quickly at R.T.; to brick red in warm room, 10 min. To purple after few hours at R.T.
17α-Methyl-17β-hydroxy-4-androsten-3α-ol	Lemon yellow to orange quickly at R.T.; to lavender to purple quickly in the warm room.

possible. When one is dealing with unknown compounds, this is of some considerable importance as indicated by the following results. When adrenal extracts were chromatographed, generally there was a UV-absorbing area at the prefront, definite but not dark. The material at the origin showed little or no absorption. When the paper was allowed to hang at room temperature for about 15 to 18 hours and then viewed again on the scanner, both the origin and prefront areas were noted to be considerably darker; that is, the UV absorption had increased. When the paper was hung in a nitrogen atmosphere for even longer periods of time, such a change did not occur. Thus, there is good indication that an oxidation took place. These phenomena were observed with both dog and rat adrenal extracts.

It was considered that perhaps a hydroxyl group with an α,β double bond was oxidized to an α,β unsaturated ketone. This may be the case; however, when 17α-methyl-17β-hydroxy-4-androsten-3β-ol was tested under similar circumstances, no UV-absorbing compound was formed. Unfortunately, no further investigation of this problem was carried out.

Testis tissue: As mentioned previously, it was standard practice after the incubation of testis tissue to extract with ether and to apply the ether-soluble material to reversed phase chromatograms. Ether-soluble material from as much as 27 gm. rat testes has been applied to one chromatogram 175 mm. wide. Most of the developed chromatograms had two definite ultraviolet-absorbing bands in the prefront region. Fig. 4 shows a tracing replica of such a chromatogram. The faster running band (more polar) contained the compounds in which there was further interest from the standpoint of androgen synthesis, such as testosterone, androstenedione, etc. It was discovered

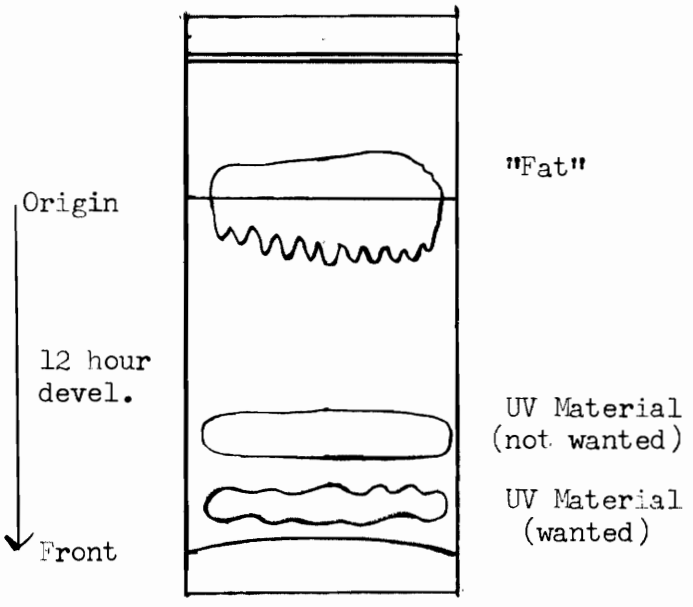

Figure 4. Replica of Reversed Phase Chromatogram (Flask S_2, Table XI, Experiment 6)

that the slower running material was formed in the absence of any testicular tissue and that it was related to the bovine serum included in the incubation medium. This UV-absorbing material was not formed when the gas phase during incubation was nitrogen, and it was not formed in the absence of nicotinamide and DPN or TPN. It has been suggested that the material might be cholestenedione arising from oxidation of serum cholesterol, but no identification has been attempted. It was formed in relatively large amounts compared with the faster running prefrontal compounds, and it was of considerable convenience to be able to obtain the desired compounds free of this material, since in the subsequent systems of chromatography, it prevented satisfactory resolution.

c. Chromatography systems of Bush.

The material which had been eluted from the prefront area of the reversed phase chromatogram was applied directly to one of two systems previously described by Bush (12). One system, called C-85, uses Skelly-Solve C as the developing phase and 85 per cent aqueous methanol as the stationary phase. It is a very useful system for separation of such compounds as testosterone, androstenedione, and progesterone. However, 17α-hydroxyprogesterone and testosterone have the same mobility (R_f 0.1) but may be separated by treating with acetic anhydride and pyridine. The former compound does not react, and upon subsequent chromatography testosterone acetate separates from 17α-hydroxyprogesterone (83).

A second system, called BC-80, utilizes 80 per cent aqueous methanol as the stationary phase and benzene-Skelly-Solve C (1:2) as the developing solvent. In this system, testosterone and 17α-hydroxyprogesterone have about the same mobility (R_f 0.6) and separation again was achieved by acetylating testosterone. It will be recalled from the resumé of the work of Slaunwhite and Samuels which preceded these experiments that 17α-hydroxyprogesterone was found to be a product of incubations of testis tissue with progesterone, and that this compound was postulated as being an intermediate in the conversion of progesterone to C_{19} androgens. It was considered quite probable that certain experimental conditions might obtain which would lead to formation of this compound while its further conversion would be blocked. Hence, it was important to determine not only the extent of conversion of progesterone to C_{19} compounds but also the extent to which 17-hydroxyprogesterone was formed. This was done by utilizing the BC-80 system in the following manner.

Material which had been eluted from the prefrontal region of the reversed phase chromatogram of an extract obtained after incubation of progesterone-21-C^{14} was concentrated at the tip of a conical tube and applied directly at the origin of a BC-80 chromatogram. After development, a 2 cm. wide strip was cut lengthwise from the center of the chromatogram. This long strip was then cut into 2 cm. squares, and each square was counted in an internal gas flow windowless counter. The amount of radioactivity in the various regions of the chromatogram could thus be determined, and a rough approximation could be made of the amount of 17α-hydroxyprogesterone formed. It was not necessary to separate testosterone from 17α-hydroxyprogesterone for this determination since testosterone formed from the incubation of progesterone-21-C^{14} was not radioactive due to the loss of the radioactive side chain carbon. If a quantitation of testosterone was desired, it was then necessary to perform an acetylation, chromatograph again, and determine testosterone acetate quantitatively by use of the Cary Ultraviolet Spectrophotometer.

7. Quantitation by Use of the Cary Recording Spectrophotometer.

Steroids such as progesterone, 17α-hydroxyprogesterone, testosterone, and androstenedione have a Δ^4,3-ketone grouping which absorbs light in the region of 238-240 mμ. This property was used as a means of quantitation. After chromatography in the BC-80 or C-85 system each paper was scanned for UV-absorbing areas, and these were marked, then cut out and eluted. If the position of a steroid could not be detected visibly, the position was marked by comparing with chromatograms already marked or by comparing with positions

of known standards which had been simultaneously chromatographed in lateral lanes. It was found that for the original chromatograms 175 mm. wide, it was much more reliable to compare lanes of equal width on which similar extracts had been chromatographed. In all cases after eluting the individual areas of these original BC-80 or C-85 chromatograms, the eluates were chromatographed again on narrow strips with standards in lateral lanes. Usually, if testosterone or 17α-hydroxyprogesterone had been eluted from a BC-80 chromatogram, they were run the second time in the C-85 system, and vice-versa. However, androstenedione and testosterone acetate were always chromatographed finally in the C-85 system, since these compounds move too close to the front in the BC-80 system.

The compounds were then eluted with cold ethanol as previously described and a similar area of paper was eluted to serve as a "paper blank". The quantity of compound was determined in a Cary Recording Spectrophotometer, Double Beam, Model 11. The absorption curve from 2200 to 2600 Å was obtained. The Allen method of calculation was applied (99) using the absorption at 230, 240, and 250 mμ. in comparison with the absorption of a known amount of steroid standard.

8. Recovery of Added Substrates.

Steroid substrates were added and incubated in the presence of Krebs-Ringer buffer and beef serum and in the absence of testis tissue. These were extracted in the same manner as the other flasks, and after chromatography the steroids were measured by the Cary Spectrophotometer. It was known from the results of extraction of radioactive progesterone that at least 5-7 per cent of the steroid substrate would remain in the aqueous phase. Another certain source of loss was in elutions (minimum of three) from paper after chromato-

graphy. There were indications also from the data on the total recovery of radioactivity and from the decreased volume of incubation medium remaining in the flask that some leakage from some few flasks occurred during the three hours of rotating the flasks in the water bath (See p. 98).

Recovery data are shown as follows:

Steroid Added	µg. Recovered	Per Cent Recovered
Androstenedione (MW 286)		
1 µMole	249	87
" "	223	78
0.1 "	22	75
Testosterone (MW 288)		
1 µMole	219	76
" "	250	87
0.1 "	22	75
17α-Hydroxyprogesterone (MW 330)		
1 µMole	231	70
" "	264	80

9. Summary of the Experimental Procedure.

Cell dispersions, homogenates, and cell fractionations of testis tissue from rats treated with human chorionic gonadotropin were incubated in a medium of Krebs-Ringer buffer and bovine serum in the presence of steroid substrates and added cofactors. Incubations were carried out in an atmosphere of oxygen or of nitrogen. The medium was then made alkaline and extracted with ether. When progesterone-21-C^{14} had been provided as the steroid substrate, the partition of radioactivity between the ether and alkaline aqueous phases was determined in order to indicate the extent of enzymatic activity in the conversion of C_{21} substrates to C_{19} products and to acid.

To obtain further information as to the nature of the products, the ether soluble material was then submitted to paper chromatography as was the ether-soluble material from incubations of non-radioactive substrates such as 17α-hydroxyprogesterone. First, the reversed phase system was used to free the

wanted steroids from less polar contaminants. After elution of the prefrontal area, resolution was carried out in either the BC-80 system or C-85 system. To separate testosterone from 17α-hydroxyprogesterone, the former was selectively acetylated and chromatographed again. Individual steroids were eluted and determined quantitatively in the Cary Spectrophotometer.

B. Individual Experiments: Procedure, Results, and Discussion

Experiment 1. Conversion of 17α-Hydroxyprogesterone to Androstenedione and Testosterone.

As further background for these experiments, it should be mentioned that as diphosphopyridinenucleotide (DPN) is known to serve as the prosthetic group for numerous biological dehydrogenases (100), so was it implicated in the conversion of progesterone to androgens by testis tissue. Slaunwhite (101) reported after preliminary experiments that testis dispersions in a nitrogen atmosphere could apparently use DPN as hydrogen acceptor for progesterone conversion. This was viewed as indicating that a fairly simple reaction sequence was occurring as previously indicated on p. 30.

There were two main purposes in performing this experiment: first, to obtain an amount of the acid resulting from scission of the progesterone side chain precedent to identification thereof, and second, to study the products of conversion by testis tissue of 17α-hydroxyprogesterone (17-OH-P) in order to see if that compound might be converted to C_{19} androgens.

Procedure: Male rats (body weight ca. 175 gm.) were hypophysectomized.[1] On the succeeding days each rat received 200 μg. ACTHAR-A by intraperitoneal injection, since it was planned to make use of the adrenal glands of these rats as well as of the testes. Two weeks later intramuscular injection of human chorionic gonadotropin (HCG) was begun; each rat received in divided doses a total of 3800 I.U. over a period of 3 weeks.

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[1] Hypophysectomies were performed by Mrs. Marva Jean Tobler. The hypophysectomized animals were maintained on a special diet of bread, milk, oranges, and carrots.

In the incubation medium, sodium succinate (0.4 mmolar) was included as well as ATP, sodium fumarate, and nicotinamide. DPN and DPNH were included as indicated in Table I. Each flask was charged with 0.93 gm. testis tissue prepared as a cell dispersion.

The progesterone series of flasks received 0.5 micromoles progesterone-21-C^{14} per flask equivalent to 400,000 counts per minute. After the incubation, each alkalinized aqueous medium was extracted with ether, and the amount of radioactivity in the ether fraction was determined. In this series, aliquots of the aqueous phase were not plated. Instead, each was acidified; then a stream of nitrogen (CO_2 free) was bubbled through and into a trap of sodium hydroxide solution. Carbonate formed from the CO_2 of the incubation medium was then precipitated as the barium salt, collected, plated, and counted for radioactivity after the techniques of Calvin, et al. (102).

The 17α-hydroxyprogesterone flasks each received 2 micromoles. The incubation medium was extracted with ether, and the steroids isolated by paper chromatography as described. Quantitative measurements of androstenedione and testosterone (as testosterone acetate) were made by means of the Cary Spectrophotometer.

It was important in this case to use some means of identification of these two steroids other than their chromatographic behavior. Thus, they were submitted to the Koenig reaction, a qualitative test of high specificity (103). The Koenig procedure has been modified by Samuels (104) to allow detection of less than a microgram of steroid.[1]

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[1] An appropriate volume of the steroid solution is introduced into a small 3 inch test tube. After the sample is dried down, it is redissolved in 0.03 ml. abs. ethanol and 0.03 ml. aq. 1 per cent $CuSO_4$. As the tubes are being cooled in an ice bath 0.2 ml. conc. H_2SO_4 is added with good

(cont. p. 63)

Results: The results are shown in Table VI.

Table VI

Conversion of Progesterone-21-C^{14} and 17α-Hydroxyprogesterone by Cell Dispersion of Testes from Hypophysectomized Rats Treated with Chorionic Gonadotropin

Incubation medium: Krebs-Ringer bicarbonate buffer and beef serum (1:1); 0.4 mmolar in ATP, fumarate, succinate; 0.04 molar in nicotinamide; 0.93 gm. testes per flask.

17α-Hydroxyprogesterone - 2 μmoles per flask.

Flask	Gas Phase	Added Cofactor 0.4 millimolar	Androstenedione μg. (Cary)	Androstenedione Koenig	Testosterone μg. (Cary)	Testosterone Koenig
1	N_2	DPN	< 5	?	5	+
2	N_2	DPNH	< 5	?	< 5	?
3	O_2	None	32	+++	8	+
4	O_2	DPN	40	+++	12	++

Progesterone-21-C^{14} - 0.5 μmoles or 400,000 counts per minute per flask.

Flask	Gas Phase	Added Cofactor 0.4 millimolar	Radioactive Counts per Minute CO_2	Radioactive Counts per Minute Ether Phase	Recovery %	Conversion %	Corrected % Conversion*
1	N_2	None	200	392,000	98	2	0
2	O_2	None	300	370,000	93	7	5
3	N_2	DPN	60	380,000	95	5	3
4	N_2	DPNH	0	391,000	98	2	0
5	O_2	DPN	500	352,000	88	12	10

* Since radioactivity of the aqueous phase was not determined directly, a simple 2 per cent correction was applied to all flasks.

(cont. from p. 61)
mixing. After the tubes are heated in a rapidly boiling water bath for 2 min., they are again cooled in an ice bath. Then 0.2 ml. 20 per cent aq. Thiocol (Hoffmann-LaRoche brand of potassium guaiacol sulfonate) is added with good mixing. Heat 2 min. in boiling water bath with gentle stirring. Cool again in ice bath. The bright green color given by androstenedione, testosterone, and testosterone esters is stable for hours in the cold. The color is read in a Beckman Quartz Spectrophotometer or a Cary Spectrophotometer, readings being taken at 630, 640, and 650 mμ. If very small amounts of compounds have been eluted from paper, an olive color may result from non-steroidal materials from the paper.

From Table VI it can be seen that there was definite conversion of 17α-hydroxyprogesterone (17-OH-P) to both androstenedione and testosterone in the presence of an oxygen atmosphere. Conversion of this substrate was not supported by DPN or by DPNH in the absence of oxygen. It does seem, however, that there was an effect of the added DPN in the presence of oxygen to increase somewhat the yield of C_{19} androgen as represented by androstenedione and testosterone. Whether 17-OH-P is on a direct route from progesterone cannot be said with certainty, however, and there is no information as to the sequence of androstenedione and testosterone synthesis. The intensity of color given by the compounds in the Koenig reaction was roughly proportional to the amounts obtained by quantitation by spectroscopy.

The best conversion of radioactive progesterone was surprisingly low in this experiment (10 per cent), since Samuels and Slaunwhite had reported conversions of 50 per cent. The addition of DPN seemed to have some small effect in both nitrogen and oxygen atmospheres. However, the conversions are so low that the significance is questionable.

Except for flask No. 1, radioactive CO_2 production tended to parallel the conversion of ether-soluble radioactivity to water-soluble radioactivity. Of the amount converted, however, only about 1 per cent could be accounted for as

$C^{14}O_2$.

From the data concerning progesterone-21-C^{14} conversion, one can calculate that in flask No. 5 about 0.05 micromoles of C_{19} steroid was produced or a total of 20 micrograms of C_{19} androgen. In the flask receiving oxygen, DPN, and 2 micromoles of 17-OH-P about 2 1/2 times this amount of C_{19} androgen (androstenedione and testosterone) was produced from 4 times the amount of substrate.

At the time this experiment was completed, it was considered as a possible explanation of the low overall conversion that the ACTH which the hypophysectomized rats received might have adversely effected enzyme formation in the testis by preventing protein synthesis. At the time of sacrifice, it was noted that few rats showed seminal vesicle stimulation: 10 out of 16 showed completely atrophied glands, the other six showing only moderate stimulation. Hence, the question of the effectiveness of the chorionic gonadotropin was also raised.

Experiment 2. Conversion of Progesterone-21-C^{14}. Response to HCG by Hypophysectomized Rats.

Hypophysectomized rats were injected daily over a period of 17 days; each rat received a total of 2200 I.U. HCG.

Each incubation flask received a testis dispersion aliquot equivalent to 0.62 gm. testis tissue except flasks 9 and 10 each of which received one pair of decapsulated testes. Each pair weighed 0.78 gm. Each flask contained 0.5 micromoles progesterone-21-C^{14} equivalent to 400,000 counts per minute except flasks 9 and 10 which contained twice this amount. The flasks were prepared in duplicate.

Results: The results are shown in Table VII.

Table VII

Conversion of Progesterone-21-C^{14} by Testis Tissue Dispersion from Hypophysectomized Rats Treated with Chorionic Gonadotropin

Incubation medium: Krebs-Ringer bicarbonate buffer and beef serum (1:1); 0.4 mmolar in ATP and fumarate; 0.04 molar in nicotinamide; 0.62 gm. dispersed testis tissue per flask except that flasks 9 and 10 each received a pair of testes; each pair weighed 0.72 gm. 0.5 μM progesterone-21-C^{14} equivalent to 400,000 counts per minute per flask. Flasks 9 and 10 received twice this amount of substrate.

Flask	Gas Phase	Cofactor 0.4 millimolar	Radioactive c.p.m. (Corrected) Ether	Alk. Aqueous	Total	Per Cent Conversion
Control*	O_2	None	308,600	21,000	329,600	--
1	O_2	None	397,800	45,000	442,800	10
2	O_2	None	368,000	47,000	415,000	11
3	N_2	None	378,000	13,900	391,900	4
4	N_2	None	378,000	23,400	401,400	6
5	O_2	DPN	366,500	39,200	405,700	10
6	O_2	DPN	404,900	38,400	443,300	9
7	N_2	DPN	430,700	17,600	448,300	4
8	N_2	DPN		(Faulty extraction)		
9	O_2	DPN	594,300	92,300	686,700	15
10	O_2	DPN	781,300	(-11,300)**	781,300	0 (-1)

* Incubated without testis aliquot. Correction factor calculated from this flask was 6 per cent.

** Correction factor of 6 per cent was 1 per cent too high for this flask.

From the foregoing data, one can conclude that the system as experimentally constituted required molecular oxygen, and that there was no effect of added DPN. This may be because this cofactor is not involved in the enzymatic processes or because the dispersed testis cells have enzyme systems not limited by the endogenous supply of this cofactor; hence, additional supplies of DPN from an exogenous source did not increase the conversion.

The best conversions were still of the order of 10 per cent. The flask duplicates agreed with each other quite well except flasks 9 and 10, each of which contained the same weight of dispersed testis tissue. These results are explained, however, upon consideration of the weights of the stripped seminal vesicles from the two rats providing the testes for these flasks.

	Weight of Stripped Seminal Vesicles	Per Cent Conversion
Rat for flask 9:	600 mg.	15
Rat for flask 10:	300 mg.	0

For the 17 rats used, stripped seminal vesicle weights varied from 145 mg. to 730 mg., the average weight being 410 mg. Thus, in this experiment there was noted a non-uniform stimulation by HCG as indicated both by the variation in weights of seminal vesicles and by the variation in enzymatic activity as indicated by the degree to which added progesterone was converted to androgen.

This experiment illustrated a point of technique; namely, that it is highly unreliable to use testes pairs of different rats in flasks receiving different addenda but rather that one should prepare the dispersion mixture and dispense aliquots therefrom.

Experiment 3. Relation Between Conversion of Progesterone-21-C^{14} and Response to HCG.

A small experiment was conducted using two hypophysectomized rats, each of which had been treated with 1700 I.U. of HCG over a period of 16 days. The incubation medium was modified in that no bovine serum was used, and that nicotinamide was 0.02 molar rather than 0.04 molar. Furthermore, DPN concentration was 0.8 millimolar rather than 0.4 mM. Each pair of testes was incubated separately in the presence of radioactive progesterone.

Results: The results are shown in Table VIII.

Table VIII

Conversion of Progesterone-21-C^{14} by Testis Tissue Dispersion from Hypophysectomized Rats Treated with Chorionic Gonadotropin

Incubation medium: Krebs-Ringer bicarbonate buffer; 0.4 mmolar in fumarate and ATP; 0.8 mmolar in DPN; 0.02 molar in nicotinamide; 0.5 μmoles progesterone-21-C^{14} equivalent to 400,000 counts per minute per flask. The gas phase was O_2.

Rat	Testes Wt. gm.	Seminal Vesicles In Situ	Seminal Vesicles Wt. Stripped gm.	Radioactive c.p.m. (Not Corrected) Ether	Alk. Aqueous	% Aqueous
1	1.38	Distended with fluid.	0.80	378,650	78,700	17
2	1.00	Little fluid	0.42	452,000	20,300	4

Once again it was seen that there was a direct relationship between the amount of accessory sex organ stimulation and the amount of conversion of progesterone-21-C^{14} by testis tissue. These data illustrate for the first

time a definite relationship between gonadotropic activity as seen by accessory sex organ stimulation and enzymatic conversion of progesterone.

The reason for the wide variation in stimulation by chorionic gonadotropin has not been elucidated. The most likely difference among the rats is in the state of hypophysectomy. However, in this type of experiment, incomplete hypophysectomy would seem not to be the answer since intact rats are known to respond well to HCG. It may be that for some reason the completely hypophysectomized rat was not responding to the gonadotropin. The question of antigenicity arises (51), but again one wonders why there should be such a variable response. Pertinent to this point is information from Mann (105) who has stated that it is his practice to give injections of gonadotropins to hypophysectomized rats in amounts of 200 I.U. every other day for 3 or 4 weeks, rather than 100 I.U. per day for 2 weeks in order to obtain uniform responses in relation to seminal fructose production. Thus, it would not seem that antigenicity is a factor here, but that in some rats there is an unexplained slow response to gonadotropin.

Experiment 4. Conversion of Progesterone-21-C^{14} by Testes from Young Non-Hypophysectomized Rats.

This experiment was carried out with testes from young intact rats which had been treated with 1600 I.U. of HCG per rat over a 14 day period.

Each incubation flask contained 0.5 μmoles progesterone-21-C^{14} equivalent to 400,000 counts per minute. Each flask received 0.73 gm. dispersed testis tissue except that flasks 1 and 2 each received a pair of testes; the weights were 1.7 gm. and 2.2 gm., respectively. The weights of the stripped seminal vesicles of these two rats were 0.51 and 0.54 gm., respectively; at sacrifice,

the vesicles were observed to be full and distended with fluid.

In the group of 24 rats the weights of the decapsulated testes varied from 1.6 gm. to 2.6 gm., the average being 1.9 gm. Seminal vesicle weights varied from 0.42 gm. to 0.69 gm., the average being 0.55 gm. Nearly all of the seminal vesicles when examined in situ were observed to be full and distended with fluid. The average body weight of the rats was 180 gm. The incubation was carried out in the standard manner.

Results: The results are shown in Table IX.

Table IX

Conversion of Progesterone-21-C^{14} by Testis Tissue Dispersion from Young Intact Rats Treated with Chorionic Gonadotropin

Incubation medium: Krebs-Ringer bicarbonate buffer and beef serum (1:1); 0.4 millimolar in ATP, fumarate; 0.04 molar in nicotinamide. 0.73 gm. dispersed testis tissue per flask except that flasks 1 and 2 each received a pair of testes, 1.7 gm. and 2.2 gm., respectively. 0.5 μM progesterone-21-C^{14} equivalent to 400,000 counts per minute per flask.

Flask	Gas Phase	Cofactor 0.4 millimolar	Radioactive c.p.m. (Corrected) Ether	Alk. Aqueous	Total	Per Cent Conversion
Control*	O_2	None	373,000*	29,400*	402,400	--
1	O_2	DPN	166,700	193,400	360,100	54
2	O_2	DPN	189,200	193,500	382,700	51
3	O_2	None	175,300	181,700	357,000	51
4	O_2	None	95,700	172,300	268,000	64
5	N_2	None	367,700	(- 7,200)**	367,700	0 (-2)
6	N_2	None	353,300	39,600	392,900	10
7	N_2	DPN	401,000	(-18,000)**	401,000	0 (-4)
8	N_2	DPN	358,000	34,000	392,000	9

* Incubated without testis tissue. Correction factor calculated from this flask was 7 per cent.

** Correction factor of 7 per cent for these flasks was too high.

It is clearly shown that the testis tissue from the treated intact rats was capable of 3 to 5 times the activity of that of the treated hypophysectomized rats under comparable experimental conditions. Added DPN did not increase the conversion. Once again nitrogen was found to be inhibitory, with or without DPN. Difficulty with emulsions during extraction was encountered with flasks 6 and 8, a fact which probably explains the high radioactivity in the aqueous phase. The low recovery in flask 4 indicates leakage during the incubation (See p. 98).

Experiment 5A. Relative Activities of Cell Suspension and Homogenate; Effects of DPN and TPN.

In the preceding experiments, it had been shown that higher conversions of progesterone could be obtained with testis tissue from young intact rats treated with HCG than with hypophysectomized rats similarly treated. A nitrogen atmosphere was found to be inhibitory; no effects of added DPN could be noted either with an oxygen atmosphere or a nitrogen atmosphere except in the first experiment with hypophysectomized rats. It was considered that either endogenous DPN was not a limiting factor in the enzymatic process or that it was not involved at all. Thus, it was necessary to resort to a method which would render DPN (or TPN) rate limiting. Such a situation could be obtained by disintegrating the cells since the "released" nucleosidases hydrolyze DPN and TPN unless inhibited by nicotinamide. Destruction in this manner of DPN or TPN in the intact cell either does not occur, occurs only very slowly, or is matched by resynthesis. Thus, in this experiment a broken cell suspension, or homogenate, was utilized.

Intact immature rats received 1500 I.U. HCG over a two-week period. At

sacrifice, all seminal vesicles were seen to be large and full of secretion.

0.03 Molar nicotinamide was used. DPN was 0.4 mmolar but TPN was only 0.3 mmolar since no correction was made for its being only 75 per cent pure. Each flask receiving a testis dispersion was supplied with 2.7 gm. testis tissue; each homogenate flask received 1.9 gm. tissue which had been homogenized for 45 seconds, using the Polytron. The substrate used was 0.5 μmoles progesterone-21-C^{14}, equivalent to 325,000 counts per minute per flask.

After the incubation, the usual extraction with ether from the alkaline aqueous phase was carried out and the radioactivity of each phase determined. The ether-soluble material was then chromatographed in the reversed phase system as previously described. The prefrontal material was eluted and chromatographed in the Bush BC-80 system. The distribution of radioactivity on the paper was determined by counting 2 cm. squares down the center of the paper as previously described. The radioactivities of the 17α-hydroxyprogesterone area and the progesterone area were summed, and the percentage of this total provided by the 17α-hydroxyprogesterone area was calculated. This was done because it was conceivable that experimental conditions might be such that formation of that compound could take place without the subsequent steps which would convert it to C_{19} androgens. The BC-80 chromatograms were not only counted; they were also viewed in the Haines UV scanner. It should be remembered that in these chromatograms, ultraviolet-absorbing material in the area of R_f 0.6 could be 17α-hydroxyprogesterone, testosterone, or other compounds of the same mobility.

Since in the different flasks there were varying amounts of progesterone 21-C^{14} remaining after the conversion, calculating 17α-hydroxyprogesterone-21-C^{14} as a percentage of the total radioactivity on the paper due to the two

areas does not give a true picture of the amount of 17α-hydroxyprogesterone-21-C^{14} formed. However, if it is assumed that progesterone and 17α-hydroxyprogesterone make up the total ether-soluble radioactivity, a condition which is closely approached, the absolute amount of 17α-hydroxyprogesterone present can be calculated. Furthermore, if one assumes that progesterone is transformed via 17α-hydroxyprogesterone to C_{19} steroids, then the radioactivity in the aqueous phase (corrected) gives a direct measure of the amount of 17-OH-P undergoing further conversion. The sum of these two values will give the total amount of 17-OH-P formed during the incubation.

Results: The results are shown in Table X (See also Figure 5). They may be summarized as follows:

1. The testis dispersion once again effected a 52 per cent conversion to C_{19} compounds in the presence of an oxygen atmosphere. Total conversion of progesterone to 17α-hydroxyprogesterone was calculated to be of the order of 90 per cent.

2. Nitrogen was inhibitory in all cases for both conversions. Since conversion to C_{19} steroids was lower than conversion to 17-OH-P (2 per cent and 7 per cent, respectively), one might argue that 17-OH-P cannot be built up in a system where further metabolism is blocked. However, the high concentrations of 17-OH-P in flasks 10, 11, 12 and 13 tend to refute this argument. It seems, therefore, that both reactions require oxygen.

3. In the flasks provided with testis tissue homogenate, a definite effect of DPN and of TPN was shown. Total conversion to C_{19} androgen and conversion to 17-OH-P did not take place in the absence of these cofactors. TPN at a lower molarity was more effective than DPN in the total conversion.

Table X

Conversion of Progesterone-21-C^{14} by Testis Tissue from Young Intact Rats Treated with Chorionic Gonadotropin

Incubation medium: Krebs-Ringer bicarbonate buffer and beef serum (1:1); 0.4 mmolar in fumarate and ATP; 0.03 molar in nicotinamide; DPN 0.4 mmolar when added; TPN 0.3 mmolar when added. Each flask contained 0.6 µmoles progesterone-21-C^{14} equivalent to 325,000 counts per minute. Each testes dispersion aliquot equivalent to 2.7 gm. tissue; each homogenate aliquot equivalent to 1.9 gm. tissue. % conversion values have been corrected by 7%. % Recovery values based on total radioactivity in water and ether phases.

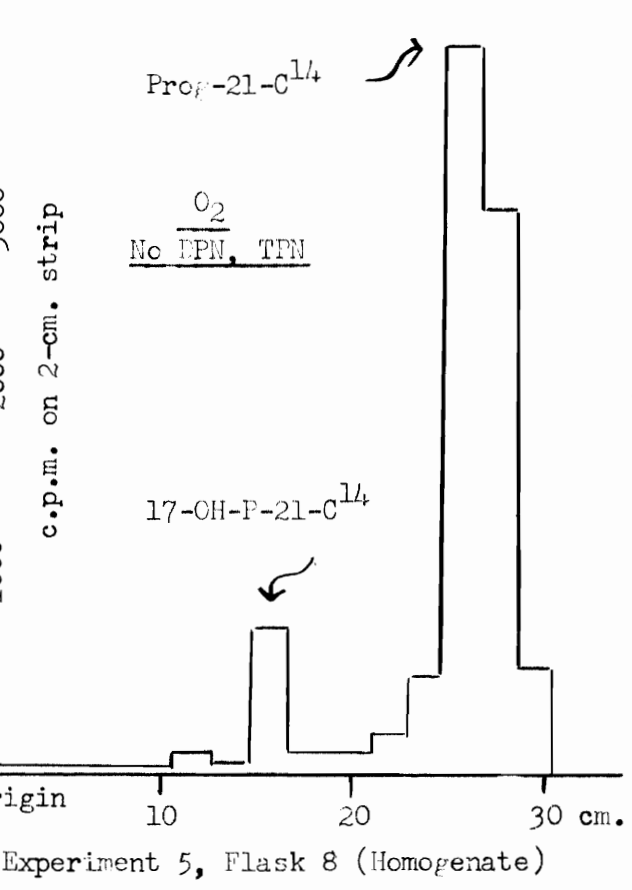
Flask	Tissue Prep.	Gas Phase	Co-factor	Extract Radioact. % Recovery	Extract Radioact. % Conversion to C_{19}	Chromatogram 17-OH-P Area % Radioactivity	Chromatogram 17-OH-P Area UV Abs.	Total µmoles 17-OH-P Formed
1	Dispers.	O_2	DPN	98	50	73	Definite	.52
2	"	"	"	94	54	74	"	.56
3	"	N_2	None	96	2	5	None	.04
4	"	"	"	96	1	5	"	.04
5	"	"	TPN	93	2	6	"	.04
6	"	"	"	93	2	Poor Chromatogram		
7	"	"	"	96	3	7	None	.05
8	Homog.	O_2	None	94	2	11	Light	.07
9	"	"	"	93	2	9	"	.06
10	"	"	DPN	88	14	60	Definite	.39
11	"	"	"	92	13	55	"	.37
12	"	"	TPN	90	21	67	"	.44
13	"	"	"	94	28	68	"	.46
14	"	N_2	None	89	3	7	None	.06
15	"	"	"	89	3	7	"	.06
16	"	"	DPN	90	4	9	"	.07
17	"	"	"	89	6	7	"	.08
18	"	"	TPN	89	6	8	Very light	.08
19	"	"	"	82	4	7	None	.06
20	"	"	"	89	6	7	"	.08
A	None	O_2	DPN	86	(8)	0	"	--
B	"	N_2	None	86	(6)	0	"	--

It may be that DPN is active after being phosphorylated to TPN.

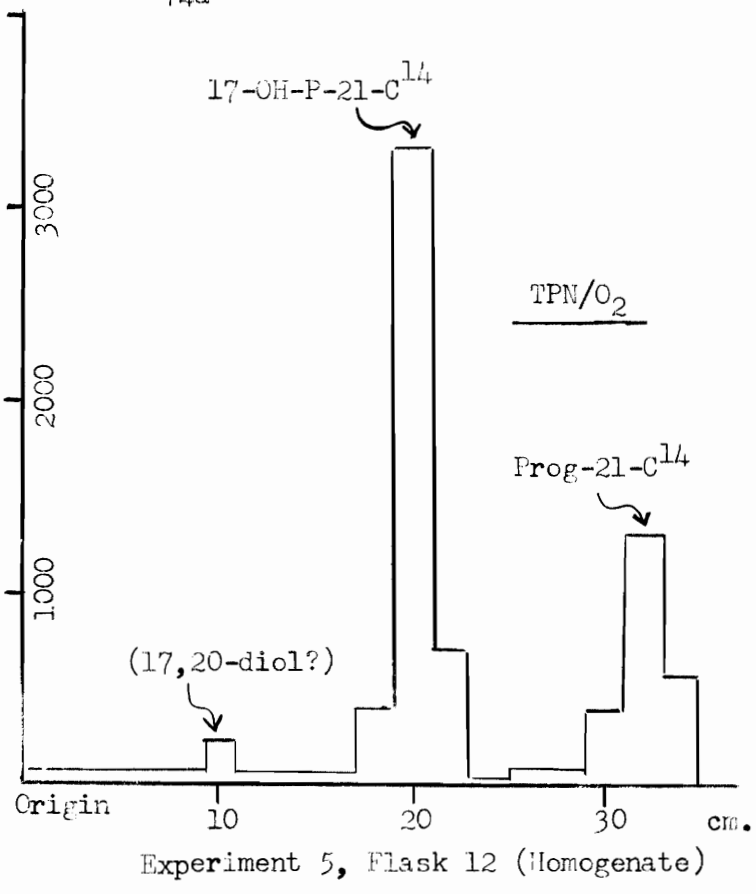
4. The homogenates were not as active as the dispersions, although there was less decrease in synthesis of 17-OH-P than in the splitting off of the side chain. The flasks receiving TPN and homogenate effected a conversion to C_{19} compounds of about 25 per cent vs. 52 per cent for the dispersion; however, conversions to 17-OH-P were 75 per cent and 90 per cent, respectively. This was due partly to the fact that there was less tissue provided in the homogenate flasks than in the ones receiving dispersion. However, the rather high conversion to 17-OH-P and the low conversion to C_{19} androgens suggests either that there was dilution effect in the homogenate which rendered the scission enzyme system less active, or that there was destruction of the latter during homogenization.

5. A very good correlation was seen between the amount of radioactivity in the 17-OH-P area and the amount of UV absorption. This absorption could have been due either to 17-OH-P or to testosterone, however, while the radioactivity was only due to 17-OH-P.

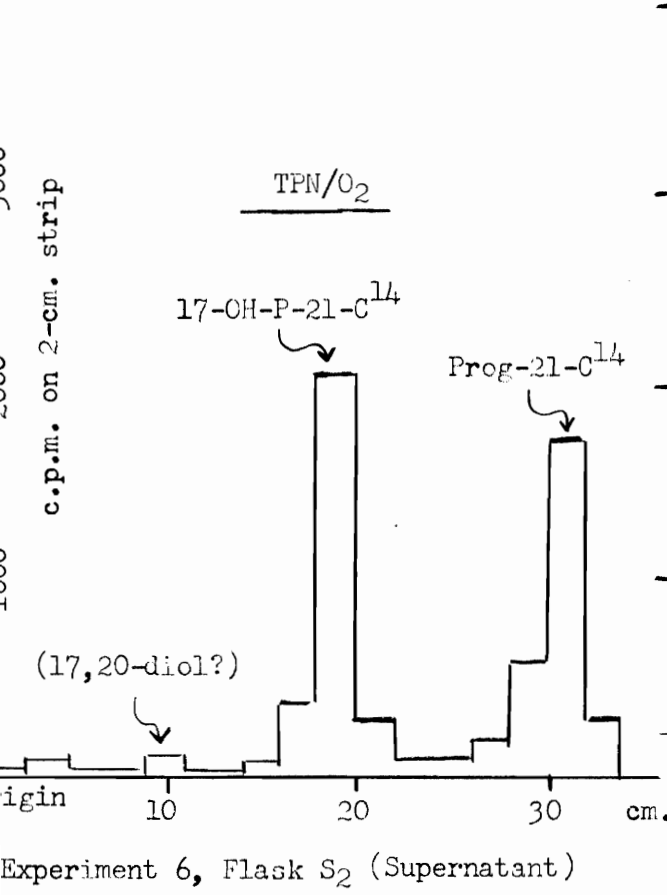
6. That there was some conversion by the homogenates in the absence of exogenous nicotinamide and DPN or TPN suggests that the endogenous cofactors took part in the reaction before being destroyed by nucleosidase activity. The slight conversion in the nitrogen atmosphere suggests either that there was residual O_2 in the flasks or that there was some factor initially present which after utilization could not be regenerated in the absence of oxygen.



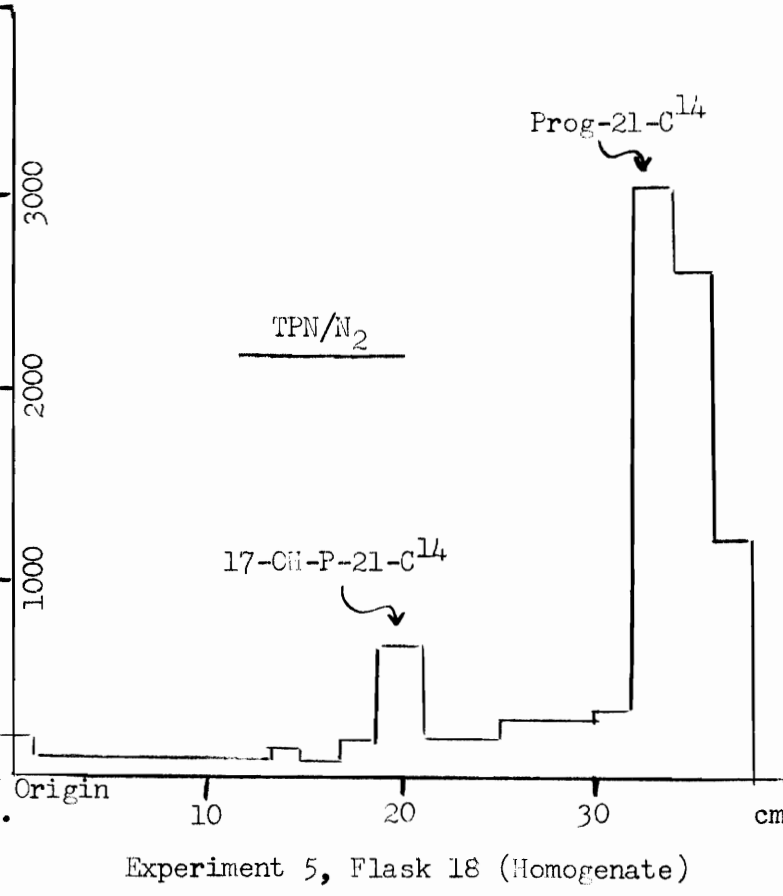
Experiment 5, Flask 8 (Homogenate)



Experiment 5, Flask 12 (Homogenate)



Experiment 6, Flask S_2 (Supernatant)



Experiment 5, Flask 18 (Homogenate)

Figure 5. Graphs of BC-80 Chromatograms

Experiment 5B. TPN and DPN as Hydrogen Acceptors for Conversion of 17α-Hydroxyprogesterone to C_{19} Steroids.

Two flasks containing testis homogenate aliquots (1.9 gm. tissue per flask) were incubated in a nitrogen atmosphere with 1 μmole (330 μg.) 17α-hydroxyprogesterone as substrate. TPN (0.3 mmolar) and nicotinamide (0.04 molar) were included. The ether extracts were chromatographed as explained. On the final chromatograms, it was not possible to identify either androstenedione or testosterone. Since it should have been possible to identify as little as 8 μg. of each steroid on the C-85 chromatogram 4.5 cm. wide, and since one could expect at least an 80 per cent recovery, one can surmise that no more than 20 μg. of the two C_{19} steroids was produced from the incubation of 330 μg. of 17α-hydroxyprogesterone; that is, the conversion was less than 6 per cent.

Since this corresponds with the amount of progesterone-21-C^{14} converted to C_{19} compounds under similar conditions (flasks 18, 19 and 20, Table X), it seems that any cofactors capable of receiving electrons from the scission system were present in very low concentration. The same result makes a simple hydrolysis unlikely.

From the experiments completed, one could draw the following working conclusions regarding the enzyme systems involved:

1. Testis homogenates are active and can be used to study both the formation of 17-OH-P and its conversion to C_{19} compounds.

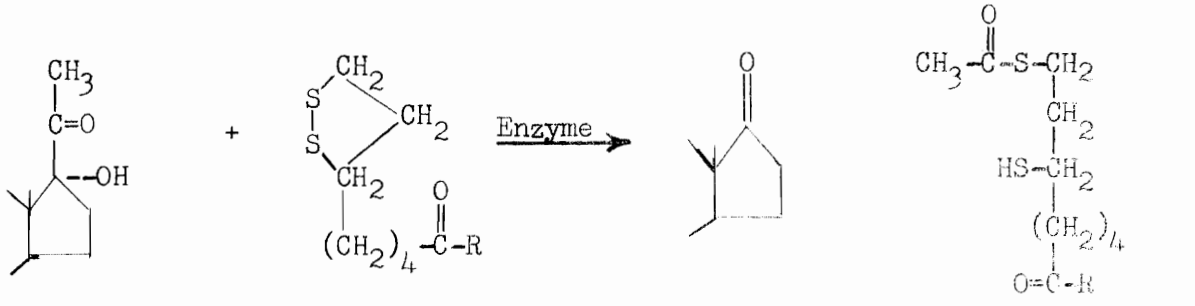
2. Either DPN or TPN is required for the conversion of progesterone to androgens, and TPN appears to be more effective.

3. In the presence of ATP, fumarate and DPN or TPN, the enzymatic systems require oxygen.

4. 17α-Hydroxyprogesterone can be converted to androgens but not by a simple hydrolytic step as previously postulated. 17α-Hydroxyprogesterone is an intermediate in progesterone conversion.

Experiment 6A. Inclusion of Riboflavin Phosphate, Lipoic Acid, Coenzyme A in Medium.

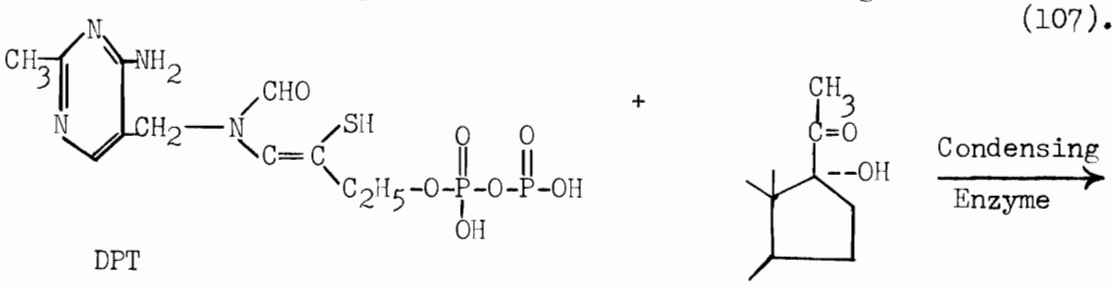
It will be recalled that previous work by Slaunwhite and Samuels on the identity of the acid resulting from enzymatic splitting of the side chain of progesterone or of 17α-hydroxyprogesterone implicated acetic acid, although this was by inference and not by proof. Since it has been shown by the results of the experiments reported here that the conversion of 17-OH-P appeared not to proceed by the direct addition of water to produce testosterone and acetic acid, other reactions were considered which could produce either androstenedione or testosterone. Thus, the following reaction was postulated (106):

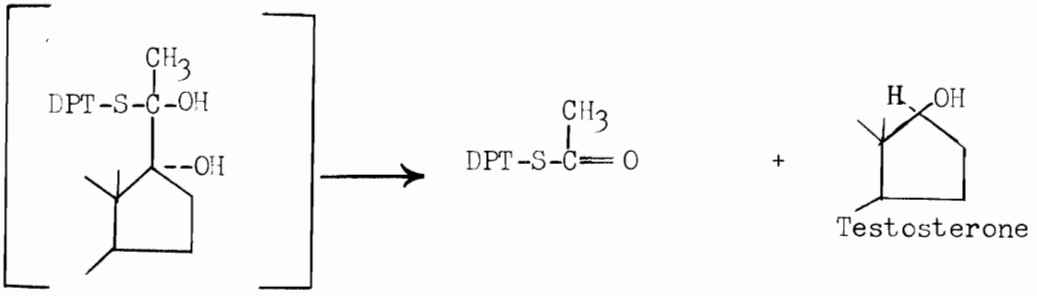


17α-Hydroxy-progesterone | Lipothiamide pyrophosphate (LTPP) | Androstenedione | Acetyl Derivative of LTPP

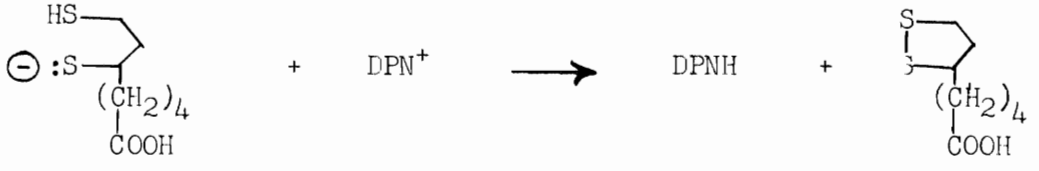
R = thiamine pyrophosphate group

Or diphosphothiamine (DPT) itself might be involved (107).





In the first case, the acetyl group would be transferred to coenzyme A, thence to hydroxyl ion to yield acetic acid. The reduced form of lipoic acid would then be oxidized by DPN or TPN to the cyclic disulfide, which could then again partake in the 17α-hydroxyprogesterone-splitting reaction. In the second case, transfer of the acetyl group would first be to lipoic acid, then subsequently as above. If such were the case, then one would expect that the addition to the medium of excess DPN would allow the reaction to be carried out effectively in the absence of oxygen. The regeneration scheme has been given as follows (106):



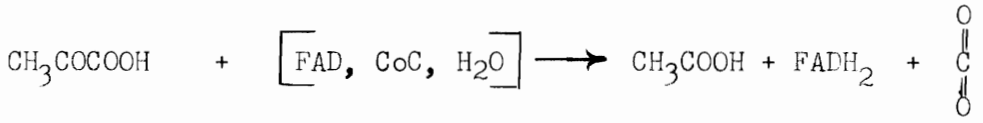
However, it has been seen in these experiments that neither DPN nor TPN

additions have supported the reaction in the absence of oxygen (or presence of nitrogen).

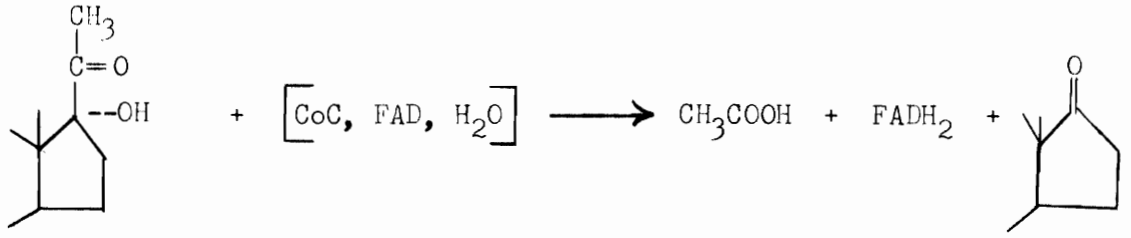
It might be that the enzyme systems of the testis utilize flavin adenine dinucleotide (FAD) as a regenerating cofactor in the following manner since FAD can participate in a 2-electron transfer:

Thus, in a nitrogen atmosphere the reaction would come to a stop since there would be no regeneration of FAD: $FADH_2$ could not be oxidized directly by molecular oxygen because there is none present nor could FAD be regenerated continually by the cytochrome system since the latter would also remain in the reduced form in the absence of oxygen.

There was also a consideration that the reaction scheme might be similar in nature to the acetate-generating mechanism of animal tissues (108) and of some bacteria (109) . For example, Proteus vulgaris has been shown to contain an enzyme system which oxidizes pyruvate to acetate and carbon dioxide without the formation of an active acetyl intermediate. The system seems to be a cocarboxylase-linked (CoC) pyruvate dehydrogenase with FAD as a cofactor.



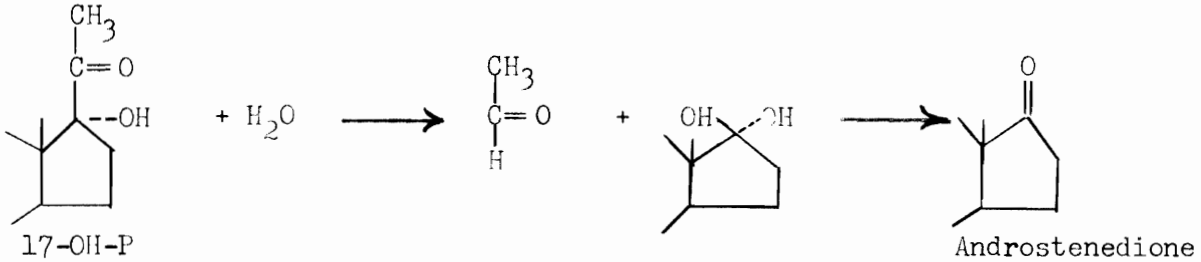
Similarly,



17-OH-P Androstenedione

One would consider, then, that in a nitrogen atmosphere this reaction would be blocked due to no regeneration of FAD.

The following reaction was eliminated by virtue of its being a hydrolytic reaction:



17-OH-P Androstenedione

The above postulations were made, of course, concerning the conversion of 17α-hydroxyprogesterone to C_{19} androgens, the specific problem of this thesis. However, it should be clearly understood that the problem of the blocking action by a nitrogen atmosphere extended to the formation of 17-hydroxyprogesterone itself since no conditions had been observed which would allow formation of that compound in the absence of conversion of progesterone to the C_{19} compounds. Furthermore, it should be mentioned

that when extracts of media showing high total conversion of progesterone-21-C^{14} were chromatographed, there was a small but definite amount of radioactivity in the region where compounds more polar than 17-OH-P are found (See Figure 5). It was considered that the radioactivity might be due to 4-pregnen-17,20-diol-3-one-21-C^{14}; this compound has been postulated as involved in the reaction sequence from progesterone (See p. 30). When extracts of media showing low total conversion were chromatographed, these radioactive "peaks" disappeared, indicating no build-up of the compound (or compounds). Thus, it could be that nitrogen blocks the formation of an intermediate between progesterone and 17α-hydroxyprogesterone. This experiment was designed to determine whether lipoic acid or riboflavin would increase the over-all conversion of progesterone.

Procedure: Immature intact rats (average body weight 230 gm.) were injected with 1600 I.U. of HCG over a period of two weeks. The incubation medium was 0.02 molar in nicotinamide. 0.3 μmole of progesterone-21-C^{14} was used as substrate. All flasks received 1.54 gm. homogenized testis tissue. At the time of this incubation no FAD or flavin mononucleotide (FMN) was available, and riboflavin phosphate was used.

Results: The results of the incubation with progesterone-21-C^{14} are shown in Table XI. The addition of coenzyme A and lipoic acid had no effect on the formation of 17-OH-P or C_{19} steroids. DPN and riboflavin phosphate did not take the place of oxygen in this system.

Experiment 6B. Relative Effects of DPN and TPN.

Since Experiment 5A had indicated that TPN was more effective than DPN in the total conversion of progesterone, this was checked using equimolar amounts of equally pure preparations (Pabst DPN: 92 per cent pure. Sigma

Table XI

Conversion of Progesterone-21-C^{14} by Testis Tissue

Incubation medium: Krebs-Ringer buffer; beef serum (1:1) 0.02 molar in nicotinamide, 0.4 mmolar in ATP and fumarate. All flasks received 1.54 gm. tissue homogenate. Each flask received 0.3 μmoles progesterone-21-C^{14} or 95 μg. equivalent to 320,000 counts per minute.

Flask	Cofactors (millimolarity 0.4 or as noted)	Gas Phase	Extract Radioactivity		Chromatogram 17-OH-P Area		Total μmoles 17-OH-P Formed
			% Recovery	% Conversion	% Radio-activity	UV Abs.	
1	DPN	O_2	96	15	72	Present	0.23
2	"	"	103	19	74	"	.24
3	DPN, Lipoic, 0.2 CoA	"	94	17	73	"	.23
4	"	"	100	19	67	"	.22
5	DPN, Riboflavin P.	N_2	98	3	<10	None	<.04
6	"	"	99	4	<10	"	<.04

TPN: 97 per cent pure). To determine whether the effect was on the formation of 17-OH-P or on its conversion to C_{19} compounds, 17α-hydroxyprogesterone was used as primary substrate with either DPN or TPN as cofactor.

The procedure was the same as in Experiment 6A, except that 1 μmole 17α-hydroxyprogesterone was used as substrate in flasks 7-12.

Results: The results of the incubation of progesterone and 17α-hydroxyprogesterone with DPN or TPN are shown in Table XII.

Table XII

Effect of DPN and TPN on Metabolism of Progesterone and 17α-Hydroxyprogesterone by Testis Tissue

Incubation medium: Krebs-Ringer buffer, beef serum (1:1); 0.02 molar in nicotinamide, 0.4 mmolar in ATP and fumarate. Flasks received 1.54 gm. tissue homogenate. Flasks 1-6 contained 0.3 μmole progesterone-21-C^{14} with an activity of 320,000 counts per minute. Flasks 7-12 contained 1 μmole 17α-hydroxyprogesterone as substrate. Gas phase was O_2. Flasks 7 and 8 contained no nicotinamide. DPN and TPN were 0.4 mmolar when added.

Flask	Cofactors mM	Substrate μmoles	Total 17-OH-P Formed μmoles	C_{19} Compounds Formed μmoles	Substrate Remaining μmoles
1	0.4 DPN	0.3 Proges.	0.23	0.045	0.07
2	"	"	0.24	0.057	0.06
3	0.4 TPN	"	0.25	0.075	0.05
4	"	"	0.25	0.084	0.05
5	0.2 DPN, 0.2 TPN	"	0.25	0.090	0.05
6	"	"	0.25	0.081	0.05
7	None	1.0 17-OH-P		0.038	0.96
8	"	"		0.059	0.94
9	0.4 DPN	"		0.108	0.89
10	"	"		0.112	0.89
11	0.4 TPN	"		0.105	0.89
12	"	"		0.129	0.87

The superiority of TPN as cofactor in the formation of C_{19} compounds when progesterone was substrate was confirmed. DPN and TPN were equally effective as cofactors for the conversion of 17α-hydroxyprogesterone to androstenedione; about twice as much androstenedione was synthesized as when DPN and TPN were absent. No testosterone (as the acetate) could be visually identified on the final chromatograms or measured in the Cary Spectrophotometer after elution from the paper.

Experiment 6C. Activity of Supernatant after High Speed Centrifugation of Testes Homogenate.

Procedure: A portion of the homogenate as prepared under Experiment 6A was suspended in the incubation medium and centrifuged at 20,000 x g. for 50 minutes. The very clear supernatant was easily decanted. It should have contained only microsomal and soluble enzymes. Aliquots equivalent to 1.47 gm. testes tissue were added to incubation flasks 1 and 2. Flasks 3 and 4 contained the original homogenate equivalent to 1.54 gm. testes.

Results: The results of the incubation are given in Table XIII. The supernatant retained considerable activity; the loss of 17-hydroxylation was not as great as the loss of the system responsible for scission of the side chain.

It was decided to investigate the products formed in the flasks containing supernatant. The androstenedione areas were eluted from the BC-80 chromatograms, and the material rechromatographed in the C-85 system. The spots showing UV absorption were eluted and measured by means of the Cary Spectrophotometer. The amount of steroid found corresponded well with the total C_{19} compounds formed as calculated from the water-soluble radioactivity.

Table XIII

Conversion of Progesterone-21-C^{14} by a Testis Tissue Supernatant

Incubation medium: Krebs-Ringer buffer; beef serum (1:1); 0.02 molar in nicotinamide, 0.4 mmolar in ATP, fumarate; 0.2 mmolar in DPN, 0.2 mmolar in TPN. Each supernatant prepared by centrifuging 1.47 gm. testis tissue as homogenate at 20,000 x g. for 50 minutes. Gas phase was O_2. Each flask received 0.3 µmoles progesterone-21-C^{14} equivalent to 320,000 counts per minute. Androstenedione has been corrected for 80 per cent recovery as determined with standards in earlier experiments.

Flask	Enzyme Source	Conversion to C_{19} Compounds %	µmoles	Androstenedione µmoles	17-OH-P µmoles	Total 17-OH-P Formed µmoles	Proges. Remaining µmoles
1	Supernat.	15	0.045	.048	0.13	0.18	0.12
2	"	16	0.048	.051	0.14	0.19	0.11
3	Homogenate	30	0.090		0.16	0.25	0.05
4	"	27	0.081		0.17	0.25	0.05

The 17α-hydroxyprogesterone-testosterone area was eluted, and the solvent evaporated. 0.4 ml. each of pyridine and acetic anhydride was added to each tube, and the reaction was allowed to proceed at room temperature overnight. One ml. of ethanol was added and the solvents removed under a stream of nitrogen. Each residue was applied directly to a C-85 chromatogram 4 cm. wide. When the developed chromatogram was examined in the UV scanner, the 17α-hydroxyprogesterone was easily visible. There was no absorption in the testosterone acetate area. The 17-OH-P areas and the testosterone acetate areas were eluted and measured in the Cary Spectrophotometer. No peak for testosterone acetate was obtained. Since no significant amounts of testosterone were

formed from 17-OH-P in Experiment 6B, it is not surprising that the supernatant was apparently inactive in this conversion.

The radioactivity of the 17-OH-P eluates was also determined. There was excellent agreement between the duplicates. The specific activity of the recovered 17α-hydroxyprogesterone-21-C^{14} was calculated to be 319,000 counts per minute and 335,000 counts per minute per 0.3 μmole while that of the added progesterone-21-C^{14} was 320,000 counts per minute per 0.3 μmole. This indicates that the 17-OH-P formed came from the added substrate with little or no contribution from endogenous sources.

Experiment 6D. Interconversion of Androstenedione and Testosterone.

It was considered that it would be helpful to know the extent of interconversion of androstenedione and testosterone by the testis tissue homogenates. The results might help in deciding whether androstenedione or testosterone is the C_{19} androgen first appearing from the scission of the C_{21} compound. The relative effects of added DPN and TPN on interconversion were also investigated.

The procedure followed was that used in Experiment 6A except that 1 μmole of either androstenedione or testosterone was used as substrate. The results are shown in Table XIV.

The data show that the testis tissue was capable of interconversion of testosterone and androstenedione. TPN was over twice as effective as DPN in the conversion of androstenedione to testosterone, but there seemed to be little difference between DPN and TPN in the conversion of testosterone to androstenedione. All duplicates were in good agreement, except that one eluate (No. 8) was apparently contaminated.

Table XIV

Effect of DPN and TPN on Interconversion of Testosterone and Androstenedione by Testis Tissue Homogenate

Incubation medium: Krebs-Ringer buffer, beef serum (1:1); 0.02 molar in nicotinamide, 0.4 mmolar in ATP and fumarate. Flasks received 1.54 gm. tissue homogenate and 1 μmole substrate. Gas phase was O_2. DPN and TPN were 0.4 mmolar when added.

Flask	Substrate	Cofactor	Androstenedione Formed μmoles	Testosterone Formed μmoles
1	Androstenedione	DPN	--	0.052
2	"	"	--	0.049
3	"	TPN	--	0.118
4	"	"	--	0.104
5	Testosterone	DPN	0.045	--
6	"	"	0.045	--
7	"	"	0.060	--
8	"	"	(UV curve illegible)	--
9	" (no testes)	"	<0.02	

Experiment 7A. Inhibition Studies with Arsenite and Mercuric Ion. Inclusion of FAD and Ascorbate in Incubation Medium.

In Experiment 6A it was found that additions of lipoic acid and coenzyme A to the incubation medium apparently had no effect on the conversion of the steroid substrate progesterone-21-C^{14}. In the absence of an effect, however,

one cannot draw the conclusion that a particular cofactor is not functional since the cofactor may not be involved in a rate-limiting step, or if it is, the endogenous concentration of the cofactor may not be limiting.

One method used frequently in biochemical studies is the use of dialysis to remove certain soluble factors from the enzyme preparation; by adding back different combinations of cofactors one can determine those which are necessary for the reaction or sequence of reactions. In the case of lipoic acid the dialysis method is not feasible since the coenzyme and apoenzyme are so tightly bound; hence, arsenite has been used in incubation media since it binds with 6,8-dimercaptooctanoic acid and thus inhibits reactions in which thioctic acid[1] is involved (109). Mercuric ion can also be used as an inhibitor for sulfhydryl groups. In general, if mercuric ion inhibits and arsenite does not, it is probable that sulfhydryl groups are involved in the reaction but thioctic acid is not. Reversal of inhibition by arsenite can be accomplished by adding lipoic acid or BAL (2,3-dimercapto-1-propanol). Mercuric ion and combinations of sodium arsenite, lipoic acid and BAL were, therefore, used in this experiment to determine more conclusively whether lipoic acid was involved in the steroid reactions.

FAD had become available and it was included in the incubation medium on the basis previously discussed. In addition, on the basis of Kern and Racker (110), ascorbic acid and Cu^{++} were added to certain flasks. These workers have described an enzyme system in yeast which oxidized DPNH. It

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[1] The names lipoic and thioctic are synonymous.

has been partially purified and found to contain a flavoprotein which required the addition of ascorbic acid for activity. The addition of ascorbic acid oxidase (AAO) caused a further three- to ten-fold increase in activity. Dehydroascorbic acid was completely inactive. Certain bacterial systems were also capable of oxidizing both DPNH and TPNH. The authors suggest that a labile one-electron oxidation product of ascorbic acid acts as the acceptor of electrons from DPNH. Similar experiments have been reported by Nason and co-workers (111). They found that oxygen was necessary for the reaction, presumably to form the labile one-electron acceptor. Cupric ions or ferric ions could replace AAO in the formation of the electron acceptor, however. Such a system could be operative here.

Procedure: Twenty-five immature and 8 mature rats each received 700 I.U. HCG over a period of 7 days. After sacrifice, a testis homogenate was prepared; each flask received 2.4 gm. testis tissue. Other addenda were as noted in Table XV.

The results show no inhibition by arsenite at the lower concentration (0.05 millimolar). There was definite inhibition at the higher concentration (20 mmolar), which was not reversed by addition of BAL. It may be that the high concentration of BAL itself was inhibiting by combining with some necessary metal cofactor such as copper. No control flask to which BAL had been added was run. The results with arsenite are, therefore, rather equivocal. The experiment should be repeated with intermediate levels in the presence of cell fractions.

Mercuric chloride (0.4 mmolar) inhibited the conversion to some extent.

Table XV

Conversion of Progesterone-21-C^{14} by Rat Testis Homogenate

Incubation medium: Krebs-Ringer buffer, beef serum (1:1); 0.04 molar in nicotinamide, 0.4 mmolar in ATP and fumarate, 0.2 mmolar in DPN, and 0.2 mmolar in TPN. Other factors 0.4 mmolar unless noted. Each flask contained 0.2 μmoles progesterone-21-C^{14} equivalent to 270,000 counts per minute. Flasks 13 and 14 were incubated with N_2 atmosphere for 3 hours, then O_2 atmosphere provided and incubated 3 hours more. Per cent conversion based on 5 per cent correction.

Flask	Gas Phase	Addenda (millimolar)	Per Cent Conversion (Corrected)
1	O_2	Control (Complete)	24
2	"	" "	21
3	"	$HgCl_2$, 0.4mM	19
4	"	" "	10
5	"	0.05 mM $NaAsO_2$	24
6	"	0.05 mM $NaAsO_2$, 0.5 mM lipoic acid	25
7	"	20 mM $NaAsO_2$	7
8	"	200 mM BAL, 20 mM $NaAsO_2$	6
9	"	1.0 mM DL-ascorbate, 0.5 mM $CuSO_4$	13
10	"	" " " "	9
11	N_2,O_2	Complete. N_2 3 hrs. then O_2 3 hrs.	15
12	"	" " "	13
13	N_2	FAD, 0.4 mM	2
14	"	1.0 mM DL-ascorbate, 0.5 mM $CuSO_4$	17

The duplicates were not in good agreement, however. This should also be repeated with cell fractions in the absence of extraneous protein.

FAD did not support the reaction in the absence of oxygen.

Flasks 11 and 12 showed that after three hours of incubation in nitrogen the enzymes were still capable of activity when an oxygen atmosphere was supplied. The conversion was about 62 per cent that of the 3 hour controls. Flask 13 indicates that conversion in these flasks (11 and 12) while nitrogen was present would have been about 2 per cent in three hours. Thus, the extended period of incubation was probably not the explanation for the conversion which did occur upon replacement of nitrogen by oxygen.

The conversion in nitrogen caused by the presence of ascorbate and cupric ion was 71 per cent that of the control. It was not matched by the conversion in the presence of ascorbate and cupric ion in an oxygen atmosphere. The explanation may be that in the presence of an oxygen atmosphere ascorbate and Cu^{++} produced hydrogen peroxide which tended to inhibit the conversion (112). One cannot say from this experiment whether the conversion was the result of the ascorbate, the copper, or both.

Experiment 7B. Cell Fractionation Studies.

Rat testis homogenate as prepared in Experiment 7A was added to Krebs-Ringer buffer and beef serum which had been made 0.25 molar in sucrose.[1] This mixture was centrifuged at 800 x g. for 10 minutes. (Two 200 ml. centrifuge bottles were used with a flat head and spun at 1600 r.p.m. in

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[1] In general, the procedure of Beyer and Samuels (62) was followed for this cell fractionation. Their methods were adaptations to adrenal tissue of those of Schneider and Hogeboom (58).

the International Refrigerated Centrifuge). The supernatant was decanted and aliquots taken for flasks 1 and 2 marked "homogenate minus nuclei". A portion was also set aside for manipulations described later in connection with flasks 10, 11, and 12.

The remaining supernatant was centrifuged at 8000 x *g*. for 10 minutes. (High speed head of the International Refrigerated Centrifuge; 15 minute run-up; center shaft speed was 2400 r.p.m.). The supernatant was poured off, and the residue was added to flasks 4 and 5 marked "mitochondria only".

The remaining supernatant ("mitochondria free"), and the supernatant taken out earlier ("nuclei free") for flasks 10, 11, and 12 were centrifuged at 100,000 x g. for 15 minutes in the Spinco Ultracentrifuge.[1] The supernatant from each centrifuge tube was decanted, and the pellet at the bottom broken up and washed out with incubation medium into an incubation flask. That from the "mitochondria-free" supernatant represented microsomes only (Flasks 8 and 9), and that from the earlier supernatant was labeled "mitochondria plus microsomes" (Flasks 10 and 11). In all cases the final incubation medium was the usual mixture of Krebs-Ringer buffer and serum which had been made 0.25 molar in sucrose. Flasks 1, 2, 3, 7, 8 and 12 also had supernatant material present from the testis tissue homogenate. Resuspension of pellet material was accomplished by violently shaking each incubation flask.

Due to various technical difficulties, not all flasks received the same

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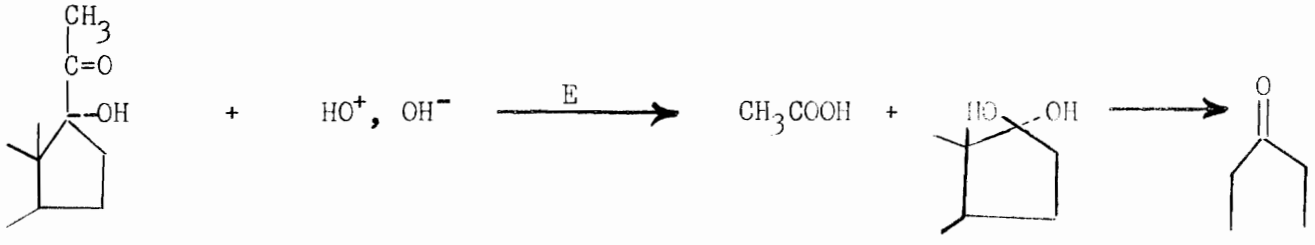
[1] Appreciation is expressed to Dr. Emil L. Smith for the use of the Spinco Ultracentrifuge and to Mr. Douglas Brown for performing the centrifugations.

equivalent of testis tissue. This is tabulated in Table XVI as testis tissue equivalents in terms of the original homogenate.

The duplicates agree fairly well except in the case of flasks 10 and 11; however, flask 11 received less microsomal material than flask 10. The results show that a clear-cut cell fractionation was not achieved. The methods used for fractionation of adrenal cortex cells are apparently not adequate for the testis. However, the separations on which one can most rely would indicate that flasks 3, 8, and 9 were free of mitochondria and that only microsomes and soluble material remained. Flasks 4 and 5 received mitochondria which were undoubtedly contaminated with microsomes since resuspension and recentrifugation was not carried out.

Experiment 8. Conversion of Progesterone-21-C^{14} by Microsomes in the Presence of TPNH; Hydrogen Peroxide; Ascorbate.

On the basis of the positive results in Experiment 7A with ascorbate and cupric ion, it was suggested[1] that hydrogen peroxide might be the agent responsible for the reaction which converts the C_{21} compound to a C_{19} compound. For example:



[1] Dr. Gordon M. Tomkins

Results: The results are shown in Table XVI.

Table XVI

Conversion of Progesterone-21-C^{14} by Testis Cell Fractions

Incubation medium: Krebs-Ringer buffer, beef serum (1:1); 0.25 molar in sucrose, 0.04 molar in nicotinamide, 0.4 mmolar in ATP, fumarate; 0.2 mmolar in DPN and 0.2 mmolar in TPN. Gas phase was O_2. Each flask received 0.2 μmoles progesterone equivalent to 220,000 counts per minute. Per cent conversions are based on 4 per cent correction.

Flask	Description of Cell Fraction Addendum	Per cent Conversion	Tissue Equivalent Based on Homogenate
1	Homogenate free of nuclei	9	0.52
2	"	10	.52
3	Homogenate free of nuclei and mitochondria	9	.49
4	Mitochondria only	8	.52
5	"	7	.52
6	Homogenate free of nuclei, mitochondria, and microsomes	6	.52
7	"	3	.52
8	Microsomes only	8	.42
9	"	8	.42
10	Mitochondria plus microsomes	17	.52
11	"	9	.42
12	Reconstitution: all but nuclei	10	.52

It was further considered that if this were the case, the addition of catalase to the incubation medium should result in an inhibition.

It was also desired to test the effect of reduced TPN (TPNH) as a cofactor. This was suggested by the work of Gillette and co-workers (113) with a rabbit liver microsomal TPNH oxidase. The enzyme was reported to be cyanide-insensitive, and hydrogen peroxide was reported to be a product of the reaction. The function of TPNH oxidase cannot be simply the production of hydrogen peroxide since peroxide generated by glucose oxidase was unable to replace the requirement for TPNH. It was noted that, as a generalization, enzyme systems catalyzing the dealkylation of alkylamines, the side chain oxidation of barbiturates, the cleavage of ethers, and the hydroxylation of aromatic rings all require TPNH and oxygen.

In the section on the general procedure, it was noted that ATP and fumarate were used as suggested by the work of Hayano and Dorfman (88) on the 11β-hydroxylation of corticosteroids. Such a requirement had not actually been demonstrated in the testis tissue incubations because these compounds had been included routinely in the medium. In this experiment, two flasks were prepared without ATP and fumarate to see if the microsomal fraction required them for conversion of the progesterone substrate.

In this experiment, it was also planned to determine again the effects of ascorbate and cupric ion.

Procedure: Eight mature rats were treated by injection of 1100 I.U. human chorionic gonadotropin over a seven-day period. At the time of sacrifice, all seminal vesicles were noted to be large and very full of

fluid. The 27 gm. of testes were homogenized in 30 ml. 0.25 molar sucrose for 1 minute by the Polytron. The homogenate was then centrifuged at 8000 x g. for 10 minutes. The supernatant was decanted carefully and submitted to centrifugation at 100,000 x g. for 1 hour. The supernatant was discarded, and the tightly packed pellets were suspended in Krebs-Ringer buffer (no beef serum) and again submitted to brief treatment with the Polytron to insure homogeneity. Aliquots were then added to the prepared flasks. Each flask received an equivalent in microsomes of 1.5 gm. testes. The incubation medium was 0.04 molar in nicotinamide but 0.2 mmolar in cofactors unless otherwise noted.

Results: The results are shown in Table XVII. This experiment was of considerable significance. Neither cupric ion and ascorbate in the absence of enzyme nor cupric ion with the enzyme but without ascorbate brought about the conversion. However, ascorbate in the presence of enzyme without added cupric ion did lead to a conversion equal to that of the control flasks. It seems rather strange that the reduced form of ascorbic acid in a reducing atmosphere (nitrogen) would be effective; however, the oxidized form of TPN was present. It may be that the oxidative splitting of the side chain requires either dehydroascorbic acid or the unknown single electron donor to bring about oxidative splitting of the side chain, and that TPN is able to oxidize the ascorbic acid to the proper degree.

TPNH was shown to be as effective as TPN. It is possible of course that the entire sequence of reactions requires both TPN and TPNH, and that if conditions for interconversion are present, either form added at the beginning of the incubation will support the conversion.

Table XVII

Conversion of Progesterone-21-C^{14} by a Microsomal Preparation of Rat Testis Tissue

Incubation medium: Krebs-Ringer buffer, (no beef serum) containing 0.04 molar nicotinamide. The buffer was 0.2 molar in ATP, fumarate or TPN, and 0.16 molar in TPNH where added. Each flask contained 0.2 μmoles progesterone-21-C^{14} or an equivalent of 220,000 counts per minute. Per cent conversions are based on a 3 per cent correction.

Flask	Enzyme	Gas Phase	Cofactors, etc. Added	Per Cent Conversion
1	Microsomes	O_2	TPNH, ATP, fumarate	9
2	"	"	"	10
3	"	"	TPN, ATP, fumarate	9
4	"	"	"	11
5	"	"	"	9
6	"	"	"	9
7	"	"	TPN; no ATP or fumarate	2
8	"	"	"	1
9	"	"	Complete*; 1 drop catalase sol.**	11
10	"	"	"	10
11	None	N_2	Complete; 1 drop 30% H_2O_2 added	2
12	Microsomes	"	"	11
13	"	"	Complete; Incubated 1 1/2 hours, then 1 drop 30% H_2O_2 added	9
14	"	"	Complete; 0.5 mmolar in cupric ion	1
15	"	"	" 1.0 mmolar in DL-ascorbate	9
16	None	"	" 0.5 mmolar in Cu^{++}; 1.0 mmolar in DL-ascorbate	0

* "Complete" means flask contained TPN, ATP, fumarate.

** This was a solution of partially purified catalase supplied by Dr. S. R. Dickman.

The deletion of fumarate and ATP had quite an effect in that the reaction proceeded to a very small extent in their absence. Whether one or both are necessary cannot be said at this point, although the matter will be discussed later.

Hydrogen peroxide in the absence of enzyme did not chemically alter progesterone-21-C^{14} as measured by conversion of the side chain to a water-soluble acid. However, conversion equal to that of the controls was obtained in the flasks containing microsomes and peroxide in a nitrogen atmosphere. However, it was noted that in both cases the addition of peroxide to the medium caused an immediate evolution of gas, presumably oxygen, which rendered the incubation atmosphere no longer oxygen-free. The added peroxide obviously did not inactivate the enzymes.

In the case of flask 13, it was planned to incubate for 1 1/2 hours, then inactivate the enzymes by boiling, then add peroxide to see if an intermediate had been resynthesized which would be acted upon by peroxide without active enzyme being present.[1] Unfortunately, the boiling at the half-way point was not done, and no conclusions can be drawn.

The addition of catalase did not inhibit the conversion. This does not indicate that peroxide is <u>not</u> involved since in some cases catalase is catalytic in hydrogen peroxide oxidations. (Example: ethanol + H_2O_2 $\xrightarrow{\text{catalase}}$ acetaldehyde + H_2O).

There is some evidence that plenty of catalase was already present in the microsomal addendum as indicated by the copious evolution of gas when hydrogen peroxide was used. This did not occur when peroxide was added to

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[1] Procedure suggested by Dr. Neil C. Davis.

the incubation mixture without the microsomal fraction present. However, it is possible that metals such as iron present in the microsomes caused the decomposition.

> Note: Flask 13 in which there was gas evolution and hence positive pressure in the flask showed a loss of 6.5 ml. (30 ml. - 23.5 ml.) after the incubation; recovery of total radioactivity was only 69 per cent vs. an average of about 95 per cent. A per cent correction by volume raises the radioactivity recovery to 90 per cent. Flask 12 was apparently tight in that both volume and radioactivity recoveries were satisfactory. A loss of similar dimensions was seen in flask 3. Such a loss does not cause an error in per cent conversion since the components of the medium are lost proportionately. The error would be serious, however, in the cases where quantitation of steroids produced is carried out.

Experiment 9. The Question of TPNH Involvement.

In the last experiment, it was seen that TPNH was as effective as TPN in the conversion of progesterone. The incubation atmosphere was aerobic, and it was quite probable that TPNH was being oxidized to TPN. Thus, there was no way of telling whether the chemical species concerned was TPN or TPNH - or both. This experiment was designed to try to prove that TPNH was involved. Use was to be made of a fact mentioned earlier; namely, that nucleosidases do not attack the reduced forms of DPN and TPN (91). Thus, if one were to incubate a homogenate in the presence of TPNH without the protection of added nicotinamide and if side chain scission were

not decreased, then one could conclude that TPNH as such is most probably involved.

Procedure: Adult rats were given injections of chorionic gonadotropin for three days. Each rat received 200 I.U. per day. The 20 gm. testes were homogenized by the Polytron in 40 ml. Krebs-Ringer buffer. The homogenate was allowed to stand at room temperature for 45 minutes with occasional mixing to allow nucleosidase activity to destroy endogenous supplies of DPN and TPN. Aliquots were then added to the incubation flasks. Each flask received 2.3 gm. testis tissue.

Results: The results are shown in Table XVIII.

Table XVIII

Conversion of Progesterone-21-C^{14} by Rat Testis Homogenate in the Presence of TPN and TPNH with and without Nicotinamide.

Incubation medium: Krebs-Ringer buffer; 0.4 mmolar in ATP, fumarate and succinate. Nicotinamide was 0.04 molar when added; TPN and TPNH were 0.2 mmolar and 0.16 mmolar when added. Each flask received 2.3 gm. testis tissue and 0.2 μmoles progesterone-21-C^{14} equivalent to 220,000 counts per minute. The conversion is based on a 5 per cent correction.

Flask	Addenda	Radioactivity (c.p.m.) Aqueous (Alk.)	Ether	Total	% Conversion
1	TPN only	39,800	204,200	244,000	16
2	" "	37,300	204,600	241,900	15
3	TPN + Nicotinamide	56,300	177,600	233,900	24
4	" "	60,500	180,000	240,500	25
5	TPNH only	36,300	194,700	231,000	16
6	" "	39,000	202,000	241,000	17
7	TPNH + Nicotinamide	51,200	183,800	235,000	22
8	" "	58,800	180,600	239,400	25

The conversions brought about by additions of TPN and TPNH were equal, both in the absence and in the presence of nicotinamide. In the presence of nicotinamide, however, conversion was 50% greater than in its absence. Accepting the assertion that TPNH is not attacked by nucleosidases, one concludes that TPNH was converted to TPN. If TPN and TPNH are both involved in the entire sequence of reactions, then TPN is concerned with a reaction "more limiting" than those in which TPNH is involved. Elucidation of this problem will probably require separation of the enzyme systems responsible.

Experiment 10. A Series of Experiments: Attempts to Identify the Acid Fragment Resulting from Enzymatic Scission of Progesterone-21-C^{14}.

From the experiments of Slaunwhite and Samuels, it had been considered that most probably the acid resulting from the conversion by testis tissue of progesterone to androgens was acetic acid. This was because it was found to be easily steam-distillable and because only about 6 per cent of the total acid was found to be formic acid as indicated by oxidation by permanganate and conversion to CO_2. Reasoning was also based on the circumstance that each progesterone molecule in going to C_{19} androgen loses two carbons, Nos. 20 and 21. It had been found furthermore by the present investigator that only about 1 per cent of the radioactivity of the total water-soluble acid resulting from incubations of progesterone-21-C^{14} was accounted for by CO_2 (Experiment 1).

Experiment 10A. It was decided to investigate again the characteristics of the acid in relation to distillation, since it was considered that an improper distillation assembly might allow the small amount of radioactive

acid (ca. 0.5 μmoles) to be carried over by the large amount of boiling water and by the "sweep" of a steam distillation.

A six-inch distilling column was prepared by packing it with glass wool to assist in refluxing and to prevent the carry-over of mist or small droplets. Both steam distillation and vacuum distillation were carried out with the water aspirator. In the latter case, the temperature of the distilling vapor did not rise over 30° C.; thus, the probability of decomposition was kept low.

The acid distillates were kept in an ice bath until the end of the distillation, when they were made alkaline. They were then taken to dryness and redissolved in 5 ml. water. Aliquots were plated for radioactivity. The distillation residues were also redissolved in 5 ml. water, and aliquots plated for radioactivity.

A basic resin column was also used. Two gm. IRA-400 filled an 8 mm. column to 7 1/2 cm. The extract aliquot was acidified to pH 4-5 and run onto the column. It was dried by aspiration, then 1 ml. water was washed through; another 1 ml. water was added. The eluate was at pH 10 to 11. Then 5 drops conc. HCl were added to 1 ml. water and poured onto the column. The eluate was at pH 1. One ml. water wash was added. The acid eluate was make alkaline; aliquots were plated and counted.

Results: The results are shown in Table XIX. They indicate clearly that the major part of the alkaline aqueous water-soluble residue from the incubation is an easily distillable acid, and the probability that it was acetic acid remained high.

An experiment was carried out, however, to see if glycolic acid could

Table XIX

Distillation of Acid Resulting from the Enzymatic Scission by Testis Tissue of Progesterone-21-C^{14}

Description	Aliquot Plated	Radioactivity c.p.m. (Total)	Per Cent of Original	
Basic Solution Control	2/5	4050†	--	
Residue Vac. Dist. of Basic Solution	2/5	3690	91	
Residue Vac. Dist. of Acid Solution	1/5	480	12	88
Distillate Vac. Dist. of Acid Solution	2/5	3080	76	
Residue Steam Dist. of Basic Solution	2/5	3920	97	
Residue Steam Dist. of Acid Solution	2/5	2210	54*	104
Distillate Steam Dist. of Acid Solution	2/5	2050	50	
Basic Eluate IRA-400 Col.		525	13	64
Acid " " " "		2085	51‡	

† About 5 per cent is accounted for by residual progesterone-21-C^{14}.

* Residue difficult to distill due to increasing volume in flask.

‡ The column was apparently not fully eluted.

distill under the same conditions. 1.26 equivalents of glycolic acid were dissolved in 15 ml. water and vacuum distilled (water aspirator) in the same type of distillation apparatus. The temperature of the distilling vapor was allowed to go to 50° C. The distillate (ice-bath) was made alkaline, after the residue was taken to dryness. The distillate required 0.06 ml. 0.1 N NaOH to titrate with phenol red as the indicator. The same volume of distilled water also required 0.06 ml. of base. The residue required 9.90 ml. to titrate; there was thus a 21 per cent loss of acidity but it did not appear in the distillate.

It was concluded that the radioactive acid from progesterone-21-C^{14} could not be glycolic acid.

Experiment 10B. On the assumption that the acid was acetic acid-C^{14}, acetic acid carrier was added to a collection of aqueous extracts. The mixture was acidified and distilled, made basic, and taken to dryness. The residue was dissolved in least amount of water, removed from the distillation flask and taken to "dryness" in a vacuum desiccator (P_2O_5). The material was then fused in a tared porcelain dish over an open flame. The fused KOAc was quickly weighed (1.9 gm.), and a weighed aliquot (175 mg.) taken. The latter was dissolved in water, and aliquots plated and counted. The KOAc (MW=98) was found to have a specific activity of 450 counts per minute per mg.; (OAc^-) would thus have an activity of 750 counts per minute per mg. (FW=59).

Cholesteryl acetate was then synthesized in the following manner. 2.9 gm. oxalyl chloride (MW=127)was dissolved in 10 ml. dry benzene. To this was slowly added 1.9 gm. of the fused KOAc. The mixture was refluxed for 1 hour in an assembly protected from moisture. This procedure for the preparation of

acetyl chloride was after Adams and Ulich (114).

The acetyl chloride formed was then quickly distilled into a solution of 21 mg. of cholesterol (purified) in 5 ml. of pyridine. The mixture was allowed to stand for 1 hour at room temperature; 10 ml. ethanol was added, and the mixture was dried down under a stream of nitrogen.

An extraction by ether was carried out, and the ether-soluble material was placed at the origin of a reversed phase chromatogram. After development, the cholesteryl acetate area was delineated by spraying a center strip 4 mm. wide with phosphotungstic acid. The area was eluted and solvent removed by a stream of nitrogen. The crystalline material (8 mg.) was dissolved in 0.4 ml. ethyl acetate and 50 lambda aliquots plated for counting. The total sample was found to have a count of 104 counts per minute. Thus, specific activity was 13 counts per minute per mg. or 98 counts per minute per mg. of acetate radicle (MW cholesteryl acetate = 445).

The cholesteryl acetate was recrystallized from ethanol; the crystalline material was separated and redissolved in 0.2 ml. ethyl acetate. Aliquots of 100 lambda were plated. The radioactivity was found to be 10 counts per minute per 1.4 mg. or 7 counts per minute per mg. The acetate radicle would thus have a specific activity of 53 counts per minute per mg. Thus, serious doubt was cast on the identification of the radioactive acid as acetic acid, since the original specific activity was 750 counts per minute per mg.

Experiment 10C. A 175 mg. aliquot of the batch of fused KOAc used in Experiment 10B was treated in the following manner. It was dissolved in 0.5 ml. ethanol in a conical centrifuge tube and 120 mg. $ZnCl_2$ (MW=136) was added. The latter dissolved and was accompanied almost at once by the

precipitation of a white, granular material. The tube was centrifuged, and the supernatant removed by pipette. 1 ml. ethanol was added to the precipitate and a slurry made. It was centrifuged again, the supernatant was removed by pipette. 1 ml. ethanol was added to the precipitate and a slurry made. It was centrifuged again, the supernatant was removed and added to the original.

The washed residue, presumed to be zinc acetate, was easily dissolved in 2 ml. water; aliquots were taken for plating and counting. The entire sample of 40 mg. gave a total activity of 120 counts per minute or 3 counts per minute per mg. (Each plate had a count of 6 counts per minute per 2 mg.).

The supernatant collection was dried down under a stream of nitrogen to a thick clear glass; this was dissolved in 1 ml. water and aliquots taken for counting.

The supernatant had a total count of 4400 counts per minute.

The zinc acetate was redissolved in water and precipitated by the slow addition of alcohol. The precipitate was washed with alcohol, redissolved in water, and aliquots plated (1 mg. per plate). The count was exactly zero.

Thus, it appears from the experiments with cholesteryl acetate and zinc acetate that the radioactive species does not have the chemical identity of acetic acid.

Experiment 10D. In spite of the experiments of Slaunwhite and Samuels, it was considered that the radioactive acid could be only formic acid. Consequently, the ether-extracted aqueous phases of flasks 1, 2, 3, 7, and 13 of Experiment 8 were combined. (These flasks had been incubated one day, extracted the next, and were distilled the next; they had been refrigerated).

This combination gave a total of about 80,000 counts per minute of easily distillable acid, as calculated from the corrected values for the individual aqueous phases.

To this mixture was added 68 mg. (1.0 millimole) sodium formate. The major volume of water was distilled off. The mixture was then acidified with sulfuric acid and distilled to dryness. Ten ml. more water was added and again distilled to dryness. The combined distillate was carefully made alkaline with a barium hydroxide solution in the presence of phenol red indicator. The water was removed in vacuo; the gas bleed was nitrogen which had been passed through a trap of KOH solution to remove CO_2.

The residue was dissolved in a minimum amount of water and transferred to a conical tube. The water was removed by a jet of nitrogen, CO_2-free. Crystallization occurred; the crystals were redissolved in 1 ml. water. Ethanol was added until cloudiness appeared; the tube was set aside to allow barium formate to crystallize. The tube was centrifuged and the supernatant drawn off by pipette; the residue was washed with ethanol and centrifuged again. The combined supernatants were measured for volume, and aliquots taken for plating and counting.

The washed barium formate was redissolved easily in 0.5 ml. water; aliquots (50 and 100 lambda) were taken for plating and counting. There were 2450 counts per minute per 13 mg., or 12,220 counts per minute per 65 mg. for the whole sample.[1] This gave a specific activity of 188 counts per minute per mg.

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[1] Note that the counts of the two fractions total 83,700; this agrees quite well with the 80,000 total calculated from the data of Table XVII of Experiment 8. See also first paragraph, p. 107.

The supernatant measured 2750 counts per minute per 3 mg. for a total of 71,500 counts per minute per 71 mg. for the whole sample.[1] Specific activity was thus 886 counts per minute per mg.

The barium formate solution was taken to dryness and easily dissolved in 0.5 ml. water. (This indicated that the precipitate was not barium carbonate as it might have been in spite of the precautions taken.) Alcohol was added to effect a precipitation. The precipitate was washed with alcohol then redissolved in water; aliquots were taken for plating and counting.

The specific activity dropped to 13 counts per minute. Thus it was concluded that the radioactive acid did not have the chemical identity of formic acid.

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[1] (See footnote - page 106).

V. DISCUSSION AND CONCLUSIONS

A. The Hypophysectomized Rat Versus the Normal Rat as a Source of Enzyme.

While the use of hypophysectomized rats injected with chorionic gonadotropin did not, under the conditions of the experiments, yield the high concentrations of enzymes expected, they revealed much about the relationships of the pituitary gland and tropic hormones to androgen synthesis. First, it is evident that development of the ability to cleave the side chain of C_{21} steroids is directly related to androgen formation following injections of chorionic gonadotropin. In those animals in which injection of the gonadotropin failed to restore significant amounts of side chain-splitting activity, there was also little development of the seminal vesicles. On the other hand, those rats which responded with significant enzymic activity also showed large seminal vesicles. This is in contrast to the findings of Huseby et al. (86) on the relationship of 3β-ol dehydrogenase activity to the androgenic function of interstitial cell tumors of the testis in mice. These workers found no correlation between this enzymic activity and androgenicity, although a very good correlation was found in the case of androgenic tumors of the adrenals. Also, Samuels (85) found that while the decrease in 3β-ol dehydrogenase activity and in size of the seminal vesicles after hypophysectomy paralleled each other, injection of chorionic gonadotropin for 4-6 days restored the enzymic function to greater

than normal levels while enlargement of the atrophic seminal vesicles did not occur with the same rapidity. Thus it seems that in the case of the interstitial cells, the restoration of the enzymes involved in conversion of progesterone to C_{19} compounds is slower than that of the enzyme converting pregnenolone to progesterone, and that these are the limiting systems in androgen formation.

Why, then, should the injection of similar amounts of chorionic gonadotropin into immature rats lead to greater enzymic activity? One possibility is that the atrophy of the interstitial cells during the period after hypophysectomy was so extensive that a considerable and variable amount of time was necessary to build up the synthetic mechanisms. In the immature rat protein synthesis in general was active, and probably some gonadotropic stimulus was already acting; thus the injected material simply speeded up a process already under way. Another possibility is that some other hormone of the hypophysis, such as the somatotropic hormone, must act synergistically with the chorionic gonadotropin to achieve maximal effect. Such hormones would be present in the immature rat but not in the hypophysectomized animal. This problem deserves further exploration.

B. Testis Dispersion vs. Testis Homogenate: Failure of the Latter to Synthesize Testosterone from Progesterone.

Attention was called earlier (p. 29) to the fact that in the work of Slaunwhite and Samuels testosterone was obtained from enzymatic action on progesterone by a preparation of dispersed testis cells. When homogenates were used, total conversion was decreased by about half, no testosterone could be identified, and an amount of material of low polarity appeared.

It was found in these present experiments that testis dispersions could form both androstenedione and testosterone when 17α-hydroxyprogesterone was added substrate but that only androstenedione could be isolated in measurable quantities from homogenates. This was true whether progesterone or 17α-hydroxyprogesterone had been used as substrate. However, it was found that testis homogenates were capable of interconverting androstenedione and testosterone. It is pointed out, however, that this interconversion was of low order (5-11 per cent) and that only 15-30 μg. C_{19} androgen was produced from the incubation of 1 μmole of substrate. Thus, if it is true that androstenedione is the first C_{19} androgen to arise from conversion of progesterone and that testosterone must arise from reduction of androstenedione, then indeed one would not expect to find more than 4-5 μg. of testosterone after incubation of progesterone or 17α-hydroxyprogesterone.

Such a low conversion to testosterone probably occurs in the homogenate because as soon as androstenedione is formed it is "diluted" by the volume of the entire medium. In the testis dispersion, androstenedione is formed inside the cell, the concentration remains relatively high, and conversion to testosterone can take place to some measurable extent. Thus, it was found in Experiment 1 (Table VI) that of 52 μg. C_{19} androgen produced, 23 per cent was testosterone. This conversion took place in the presence of added DPN. It might have been even higher had TPN been added, since TPN was more effective in the conversion of androstenedione to testosterone than DPN (Table XIV, p. 86).

The above theory, that androstenedione is formed first from C_{21} steroids then converted to testosterone is also supported by the finding that in one

experiment the amount of progesterone-21-C^{14} converted to C_{19} androgen was almost quantitatively accounted for by the amount of androstenedione recovered (Table XIII, p. 84).

Discrepancies, however, with the Slaunwhite and Samuels observations remain, and the theory does not explain the appearance of the material of low polarity. In this connection, Mason (115) has found that when rat testis tissue dispersions were incubated with radioacetate, free radiotestosterone could not be identified. Instead, considerable radioactivity was found in the R_f 0.7 area of a Bush C-85 chromatogram (R_f testosterone = 0.1). When the radioactive material was eluted and treated with alcoholic KOH, a substance tentatively identified as testosterone was obtained.

These findings, although obtained with a dispersion rather than a homogenate and with acetate as a precursor, suggest that the material of low polarity which Slaunwhite and Samuels encountered might be a testosterone derivative of similar properties. Furthermore, it means that testosterone might have been present as a non-polar derivative in the experiments of this thesis. In such a case, they would have chromatographed in the general prefrontal area of the BC-80 chromatograms, not further examined.

It was found generally that the most active homogenates were about half as active as the most active dispersions in terms of total conversion of added progesterone-21-C^{14} substrate to C_{19} compounds and acid. However, there was not as much of a decrease in production of 17α-hydroxyprogesterone. It was shown in Experiment 5, for instance, that the dispersion metabolized 90 per cent of the progesterone added, and the homogenate 78 per cent in spite

of the fact that there was 30 per cent less testis tissue in the homogenate flasks. These results show that homogenization decreased the activity of the enzyme system responsible for C_{19} production more than it decreased the activity of the 17α-hydroxylating system. Whether such a decrease is due to destruction of enzyme or cofactor or to dilution of components of the enzyme system is not known.

C. 17α-Hydroxyprogesterone as an Intermediate.

The results, thus far, show that 17-OH-P can be an intermediate between progesterone and the C_{19} androgens, but do not prove an obligatory role. Slaunwhite and Samuels (83) showed that testis tissue can convert progesterone to 17-OH-P, and that testosterone and androstenedione also were products of progesterone metabolism in the same system, but they did not demonstrate that 17-OH-P itself could be converted to the androgens. In the present experiments this conversion was brought about by testis tissue. Further, when homogenates, which could not convert androstenedione to free testosterone, were used as source of enzyme, there was a larger proportionate accumulation of 17-OH-P than when the conversion was carried to testosterone by cell dispersions.

While these results indicate that 17-OH-P is on one of the routes by which androstenedione and testosterone are formed from progesterone, it does not establish this as the only pathway. While the results of the experiments reported here do not support glycolic acid as the two-carbon fragment split off, it is possible that hydroxylation of carbon 21 might occur first, followed by 17-hydroxylation. In this case desoxycorticosterone and Reichstein's substance S would be the intermediates. These compounds have

never been observed in the extracts after incubation, but they might be so rapidly converted to C_{19} compounds that the concentrations would be too low for identification. Since neither of these compounds has been used either as substrate or as trapping agent this possibility cannot be ruled out.

D. Role of Phosphonucleotides.

When testes homogenates were used, additions of either DPN or TPN were necessary for conversion. TPN was shown to be more effective for the conversion of progesterone to 17α-hydroxyprogesterone and for the conversion of progesterone to androgens. Since DPN and TPN were equally effective in the conversion of 17α-hydroxyprogesterone to androstenedione, it was considered that the TPN effect on the total conversion of progesterone to androgen was by virtue of its role in the production of 17α-hydroxyprogesterone.

Since TPNH and O_2 have been implicated in enzymatic hydroxylations, an attempt was made to see if TPNH could be involved in a rate-controlling step in the conversion. TPN and TPNH were included in an incubation of homogenate in the presence and absence of nicotinamide (Experiment 9), on the basis that nucleosidases do not attack TPNH. The results showed that TPN and TPNH without nicotinamide were equally effective, and that TPN and TPNH with nicotinamide were equally effective. Protection by nicotinamide allowed greater conversion. Thus, no conclusions could be drawn. However, pertinent information is available from the results of Experiment 8, Table XVII, p. 96. It had been previously found[1] that certain enzymatic hydroxylations brought about by TPNH and O_2 can be inhibited by catalase; hence, the active factor

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[1] Dr. Gordon M. Tomkins. Personal communication.

was considered to be hydrogen peroxide. Since in the present experiments catalase was found not to be an inhibitor of the progesterone conversion, it is considered probable that the reaction TPNH + O_2 ⟶ HOOH ⟶ hydroxylation is not involved.

TPN was found to be considerably more effective than DPN in supporting conversion of androstenedione to testosterone by testis homogenate. This difference was not seen in the opposing conversion. Since one assumes that androstenedione is converted to testosterone by reduced nucleotide, a limiting step may have been encountered in the production of DPNH.

In the cases cited where DPN is active, one is not certain whether it is active as DPN or by phosphorylation to TPN. It will be recalled that the incubation medium contained added ATP.

When one is comparing effects of compounds such as DPN and TPN added to mixtures such as homogenate, one is not certain that the more positive effect of one factor is not due to the increased destruction of the other. However, in these experiments an amount of cofactor thought to be in considerable excess was added: 10 μmoles cofactor vs. 0.3-1.0 μmoles substrate.

E. Role of ATP and Fumarate.

Since it was found that when ATP and fumarate were deleted from the incubation medium a microsomal preparation of testis tissue was unable to convert progesterone-21-C^{14} to C_{19} steroids even in the presence of TPN and oxygen, it appears that one or both are involved in the reaction. Unfortunately there was not time to determine whether one or both were essential. However, in a study of the 17α-hydroxylating system of beef testis, Plager (116) has found that ATP is not essential when TPN is included in the incubation medium rather than DPN. In their study of the 11-hydroxy-

lating system of the beef adrenal, Hayano and Dorfman (119) found that TPN would replace DPN + ATP, but fumarate and O_2 were also necessary. Only malate and succinate could replace fumarate (88), being respectively 90 and 75 per cent as effective. The 4-carbon dicarboxylic acids were postulated to be involved in hydrogen transfer. On the basis of these observations it might be expected that in the presence of TPN, fumarate was the essential cofactor rather than ATP.

F. Involvement of Lipoic Acid and Coenzyme A.

Addition of lipoic acid and coenzyme A to the incubation medium did not bring about an increase in conversion over that of the control. Addition of mercuric chloride (0.4 mmolar) to a homogenate produced some inhibition (about 35 per cent) but the mechanism of the inhibition is not known to be through sulfhydryl groups. There was considerable testis tissue present, however, and it is probable that a higher inhibition at a lower concentration of mercuric ion would be obtained with a microsomal preparation.

Arsenite at a millimolarity of 0.05 was not inhibitory. However, at a millimolarity of 20 it was quite inhibitory (ca. 73%). (In studying the enzymes of the pyruvate oxidase system of P. vulgaris, Moyed and O'Kane (109) used a concentration of 30 mmoles per liter). The finding that arsenite inhibition can occur suggests, but does not prove, that lipoic acid might be involved in the conversion of progesterone. A postulation of such an involvement was presented on page 76. If lipoic acid is involved, presumably a lack of oxygen breaks some link in the chain of re-oxidation of reduced lipoic acid. In case the inhibition resulting from the addition of arsenite was due to its conversion to arsenate, one would still postulate a necessary involvement of sulfhydryl groups.

The failure of 2,3-dimercaptopropanol (BAL) to reverse the inhibition to any extent was rather surprising. However, it was present in considerable excess and may have played its own role in the inhibition by combining with a necessary metal such as copper or manganese. Addition of BAL alone was not tried.

In the future, experiments with partially purified enzyme systems may allow measurement of the accumulation of acetyl lipoic acid, if indeed such a compound is formed (118). Like other acyl mercaptides, acetyl lipoate yields a hydroxamic acid with hydroxylamine and, unlike acetyl phosphate, is heat stable.

G. Lack of Conversion in Nitrogen Atmosphere; Replacement of the Need for Oxygen.

At the beginning of these experimentations, a personal communication from Dr. Roy Slaunwhite had indicated that added DPN would support the conversion of progesterone in a nitrogen atmosphere. Consequently, it was anticipated that most of the experimental effort could be expended on fractionation of the enzyme system directly responsible for the scission of the C_{21} precursor. However, when it was found that neither DPN nor TPN could support the reaction in the absence of oxygen, it was felt that it was of considerable importance to find a factor which could be added to allow conversion in the absence of oxygen, although it was recognized that molecular oxygen might be directly involved in a hydroxylation step.

Additions of riboflavin phosphate and flavin adenine dinucleotide (FAD) were not effective in replacing oxygen. No additions of cytochrome c were made.

It was finally discovered that a rat testis homogenate preparation was able to convert progesterone in the absence of oxygen when DL-ascorbate (1.0 mmolar) and cupric ion (0.5 mmolar) were included in the medium. (Other inclusions were ATP, DPN, TPN, and fumarate). (Experiment 7A, Table XV). It was determined that the conversion in the presence of ascorbate and Cu^{++} did not occur in the absence of enzyme (Experiment 8, Table XVII). The addition of cupric ion was not necessary, since it was found that addition of ascorbate alone to a microsomal preparation supported the conversion in a nitrogen atmosphere. The medium also contained TPN and fumarate.

It was surprising to find ascorbic acid active in an atmosphere of nitrogen. Enzyme systems in yeast and bacteria which require ascorbic acid for activity of a flavoprotein DPNH (and TPNH) oxidase have also required oxygen (110, 111).

Ascorbic acid without copper was not added to an incubation medium with an oxygen atmosphere. In the flasks where both ascorbate and copper were present with an oxygen atmosphere, the conversion was less than the control and less than when ascorbate and copper were present with a nitrogen atmosphere (Experiment 7A, Table XV). It is suggested that either there was destruction by a non-enzymatic oxidation of ascorbate in the presence of Cu^{++} and O_2, or that there was inhibition by cupric ion. This could have been checked by the addition of cupric ion alone to an otherwise complete mixture to check for inhibition, but it was not attempted. It is known that the inhibitory agent was not hydrogen peroxide, since additions of peroxide did not decrease the conversion (Experiment 8, Table XVII).

The role of ascorbate may be in the formation of a labile one-electron oxidation product as postulated by Kern and Racker (110) and by Nason and

co-workers (111). In the conversion of progesterone by testis tissue, it may not be directly linked to DPNH or TPNH. Much more experimental work needs to be done with ascorbic acid in this connection.

H. Identity of the Radioactive Acid.

It was rather surprising when the radioactive acid resulting from the enzymatic scission of the side chain of progesterone-21-C^{14} was not identified as acetic acid. It was felt that its distillation characteristics had indicated it to be either formic acid or acetic acid, and previous work by Slaunwhite and Samuels had suggested that only about 6 per cent of the volatile acid was formic. In the experiments reported here, addition of formic acid carrier and precipitation as barium formate also indicated that the major portion was not formic acid-C^{14}.

In considering the biochemical reactions possible, it was thought that the acid might be glycolic or glyoxylic. The former did not distill under the conditions applied to the distillation of the radioactive acid, however. No experiments were carried out with glyoxylic acid, but it is considered that it would also not be volatile under the conditions here applied. Furthermore, on heating with alkali, glyoxylic acid may disproportionate into glycolic acid and oxalic acid, neither of which is volatile. Oxalic acid and glyoxylic acid are also excluded on the basis of the permanganate oxidation since they would be oxidized to CO_2.

It was considered that the acid might be a sulfur analog of either formic or acetic acid. However, these thioacids are quite unstable, particularly as sodium salts.

The three-carbon acids, propionic, acryllic, pyruvic and lactic, were considered unlikely since the three-carbon chain could not be obtained intact

without rupture of the steroid nucleus. These acids would, however, have significant vapor pressures at the boiling point of water. It would be possible for pyruvic acid to be formed by carboxylation of an acetyl fragment. If this were the way in which the volatile acid came about, however, one would expect that it would be converted to acetic acid and CO_2 on treatment with permanganate, and it was the acid distilled off of such an oxidation that Slaunwhite and Samuels found not to distribute in a counter-current distribution system along with acetic acid. Conclusive experiments to rule out this possibility need yet to be done.

Under the conditions used in the distillation from acid, it would have been expected that acetoacetic acid, which might well have been formed from two acetyl radicles in the crude system, would have broken down to yield acetone. Since the product of such a distillation was shown still to be an acid, this hypothesis seems unlikely. The fact that addition of coenzyme A did not increase the formation of C_{19} compounds also argues against this possibility, since the experiments with 17-OH-P as substrate indicate that the scission of the side chain is probably the limiting reaction in the system. At present, it seems that the best approach to identification of the acid is to obtain enough of the material from incubation with microsomes, thus eliminating many possible secondary reactions, to carry out a systematic structure study.

I. Cell Fractionation Studies.

The methods used did not give clear-cut separation of cell components of the testis. Careful study will have to be made in this area to determine the best methods for the preparation of the various cell fractions. However, the following points are pertinent:

1. It might have been of some advantage to use testes from HCG-stimulated rats in which the seminiferous epithelium had been allowed to atrophy. Thus, much "contaminating" material would have been absent at the beginning.

2. The initial centrifugation of the homogenate, prepared in 0.25 molar sucrose at 800 x g. for 15 minutes seemed to be satisfactory. The second centrifugation at 8000 x g. should have been repeated at least twice on the resuspended mitochondria to aid in preparation of mitochondria less contaminated by microsomes.

3. The supernatant from the above treatment should have been examined microscopically for presence of mitochondria and then centrifuged at 100,000 x g. for 1 hour to prepare a microsomal pellet and to free the supernatant of microsomes. The Leydig cells of the testis are known to be highly lipoidal, and it is reasonable to assume that the microsomes are also high in lipid and lipoprotein. Therefore, their density would be rather low, and centrifugations at lower speeds or at the higher speed for shorter times are not sufficient to free the supernatant of microsomes. If the microsomal fraction alone is desired, the original homogenization might be carried out in distilled water so that the density of the final supernatant is as low as possible. This would assist in removing the low density microsomes from the medium.

From the data and discussion previously provided (p. 91), it seems fairly certain that the enzyme systems involved in the conversion or progesterone are not located in the soluble enzyme fraction nor in the mitochondria but in the microsomes. There was a suggestion that conversion by both mitochondria and microsomes was greater than by microsomes alone.

VI. SUMMARY

1. A new system of chromatography has been described which allows resolution of the "less polar steroids" such as cholesterol and congeners. It is a reversed phase system utilizing odorless kerosene as the stationary phase, and 60 per cent aqueous n-propanol as the developing phase. It has proved extremely useful in separation of "fat" and other unwanted compounds from more polar ones for which further resolution is desired.

2. A new color reagent, phosphotungstic acid, has been presented. It has proved useful in the location and identification of certain steroids having a Δ^4,3-ol or a Δ^5,3-ol grouping.

3. Incubation of preparations of testes from hypophysectomized rats treated with human chorionic gonadotropin have shown a correlation between the amount of gonadotropic stimulation, as indicated by the reversal of atrophy of the seminal vesicles, and the amount of conversion <u>in vitro</u> by the testes of progesterone-21-C^{14} to C_{19} androgens.

4. Testis tissue from normal immature rats treated with HCG has shown a much higher capacity (4-5 times) to convert progesterone to androgen than testis tissue from hypophysectomized rats similarly treated.

5. During the conversion of progesterone-21-C^{14} to C_{19} androgen, a radioactive acid was produced. It has the distillation characteristics of formic acid or acetic acid but appears to be neither as indicated by isotope

dilution experiments. The chemical identity of the acid remains unknown.

6. It was shown that rat testis tissue was able to convert 17α-hydroxyprogesterone to androstenedione and testosterone. Whether 17-OH-P is an obligatory intermediate in the conversion of progesterone to C_{19} androgens has not been determined with certainty, but it would seem to be so.

7. The data of these experiments indicate that androstenedione is the first C_{19} compound produced from a C_{21} compound and that testosterone arises from reduction of androstenedione. Testis tissue was shown to be able to interconvert androstenedione and testosterone with a dependence of mediation by phosphonucleotides.

8. Production of both androgen and 17α-hydroxyprogesterone from added progesterone and conversion of added 17-OH-P to androgen was blocked when a nitrogen atmosphere replaced oxygen in the incubation flasks. It was considered that 17-OH-P is probably not converted to androgen by simple one-step hydrolysis as originally postulated.

Conversion in the presence of a nitrogen atmosphere was accomplished by both a testis homogenate and a testis microsome preparation when ascorbate was added to the incubation medium also containing TPN, fumarate, and ATP. The role of ascorbate is unknown.

9. Added DPN and TPN were necessary for conversion of progesterone and for conversion of 17α-hydroxyprogesterone by homogenates. TPN was more effective than DPN in the total conversion of progesterone while DPN and TPN were equally effective in the conversion of 17α-hydroxyprogesterone. It is postulated that TPN is a cofactor in the synthesis of 17α-hydroxyprogesterone.

10. Inhibition of the conversion of progesterone was accomplished by the

inclusion of mercuric ion in the incubation medium. Inhibition was also obtained by the inclusion of arsenite. It is suggested that lipoic acid might be involved in the scission of 17α-hydroxyprogesterone to produce C_{19} androgen.

11. The intracellular enzymes responsible for the production of androgens from progesterone seem to be located in the microsomes. Non-involvement of the mitochondria was not established, however.

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VII. BIBLIOGRAPHY

1. David, K., Dingemanse, E., Freud, J., and Laqueur, E., Z. physiol. Chem. 233: 281 (1935).

2. Smith, P. E., Anat. Record 32: 221 (1926).

   J. Am. Med. Assoc. 88: 158 (1927).

   Am. J. Anat. 45: 205 (1930).

3. Moore, C. R., and McGee, L. C., Am. J. Physiol. 87: 436 (1928).

4. Greep, R. O., Fevold, H. L., and Hisaw, F. L., Physiol. 65: 261 (1936).

5. Pregl, F., Ber. 44: 553 (1911).

6. Butenandt, A., Z. angew. Chem. 44: 905 (1931).

7. Dorfman, R. I., "Biochemistry of Androgens", p. 467, The Hormones, Vol. I. Acad. Press Inc., New York (1948).

8. Goppelsroeder, F., Kolloid- Z. 4: 23,94,191,236,312 (1909).

9. See Principles and Practices of Chromatography. Zechmeister, L., and Cholnoky, L., John Wiley and Sons Inc., New York (1943).

10. Consden, R., Gordon, A. H., and Martin, A. J. P., Biochem. J. 38: 224 (1944).

11. Zaffaroni, A., Recent Progress in Hormone Research, Vol. VIII, p. 51 (1953).

12. Bush, I. E., Biochem. J. 50: 370 (1952).

13. Hunter, J., in Biological Action of Sex Hormones by Burrows, Cambridge Univ. Press (1949).

14. Berthold, A. A., Arch. Anat. w. Physiol. u. wissensch. Med. 42-46 (1849).

15. Loewy, A., Ergebn. der Physiol. 2: 130 (1903).

16. Selye, H., Textbook of Endocrinology, Universite de Montreal, Canada (1947).

17. Pézard, A., Compt. Rend. Acad. Sci. 153: 1027 (1911).

18. Gallagher, T. F., and Koch, F. C., J. Biol. Chem. 84: 495 (1929).

19. Addison, T., "On Constitutional and Local Effects of Disease of Suprarenal Capsules". M. Classics 2: 244 (1937).

20. von Mehring, J., and Minkowski, O., Arch. exptl. Path. Pharmakol. 26: 371 (1890); 31: 85 (1892).

21. Bayliss, W. M., and Starling, E. H., J. Physiol. 28: 325 (1902); 29: 174 (1903).

22. Starling, E. H., "The Chemical Correlation of the Functions of the Body", Lancet (Part II), 339 (1905).

23. Takamine, J., J. Soc. Chem. Ind. 20: 746 (1901).

24. Kendall, E. C., J. Am. Med. Assoc. 64: 2042 (1915).

25. Banting, F. G., and Best, C. H., J. Lab. Clin. Med. 7: 464 (1921-22).

26. Macleod, J. J. R., J. Metabolic Research 2: 149 (1922).

27. Collip, J. B., Trans Roy. Soc. Canada 16: 3 (1922).

28. Abel, J. J., Geiling, E. M. K., Rouiller, C. A., Bell, F. K., and Wintersteiner, O., J. Pharmacol. 31: 65 (1927).

29. Aschheim, S., and Zondek, B., Klin. Wochschr. 6: 1322 (1927).

30. Doisy, E. A., Veler, C. D., and Thayer, S. A., Am. J. Physiol. 90: 329 (1929).

31. Butenandt, A., Naturwisscenschaften 17: 878 (1929).

32. MacCorquodale, D. W., Levin, L., and Thayer, S. A., J. Biol. Chem. 115: 435 (1936).

33. McGee, L. C., Biological Activity of Testicular Extracts. Ph.D. Dissertation, Univ. of Chicago (1927).

34. Loewe, S., Voss, H. E., Lange, F., and Wähner, A., Klin. Wochschr. 7: 1376 (1928).

35. Butenandt, A., Tscherning, K., Z. physiol. Chem. 229: 167 (1934).

36. Butenandt, A., Tscherning, K., Z. physiol. Chem. 234: 224 (1935).

37. Dingemanse, E., Freud, J., and Laqueur, E., Nature 135: 184 (1935).

38. Gallagher, T. F., and Koch, F. C., Endocrinology 18: 107 (1934).

39. Fieser, L. F., and Fieser, M., Natural Products Related to Phenanthrene, p. 368, Rheinhold Publ. Corp., New York (1949).

40. Tschopp, E., Nature 136: 258 (1935).

41. Butenandt, A., and Hanisch, G., Z. physiol. Chem. 237: 89 (1935).

42. Ruzicka, L., J. Am. Chem. Soc. 57: 2011 (1935).

43. Dorfman, R. I., and Ungar, F., The Metabolism of Steroid Hormones, p. 10, Burgess Publ. Co. Minneapolis (1953).

44. West, C. D., Hollander, V. P., Kritchevsky, T. H., Dobriner, K., J. Clin. Endocrinol. 12: 915 (1952).

45. Dorfman, R. I., "Steroid Hormone Metabolism", The Hormones Vol. III, Academic Press, New York (1955).

46. Baggett, B., Engel, L. L., Savard, K., Dorfman, R. I., Federation Proc. 14: 175 (1955).

47. Bouin, P., and Benoit, J., "Quels sont, dans le testicele, elements cellulaires qui elaborent l'hormone sexuelle"? Proc. 2nd Internat. Congress for Sex Research, p. 264, London (1930).

48. Turner, C. D., General Endocrinology, W. B. Saunders Co., Philadelphia (1948).

49. Zondek, B., and Aschheim, S., Klin. Wochschr. 6: 248 (1927).

50. Li. C. H., and Evans, H. M., "Chemistry of Anterior Pituitary Hormones", The Hormones, Vol. I, 631 (1948).

51. Evans, H. M., and Simpson, M. E., "Physiology of the Gonadotrophins", The Hormones, Vol. II, 351 (1950).

52. Cowie, A. T., and Folley, S. J., "Physiology of the Gonadotropins and the Lactogenic Hormone", The Hormones, Vol. III, 309 (1955).

53. Conference on "Biology of the Testes", Ann. N. Y. Acad. Sci. 55: 543-742 (1952).

54. Aschheim, S., and Zondek, B., Klin. Wochschr. 6: 1322 (1927).

55. Maddock, W. O., Epstein, M., Nelson, W. O., Ann. N. Y. Acad. Sci. 55: 657 (1952).

56. Gomori, G., Proc. Soc. Exp. Biol. & Med. 58: 362 (1945).

57. Linderstrøm-Lang, K., "Distribution of Enzymes in Tissues and Cells", Harvey Lectures, 34: 214 (1938-39).

58. See Schneider, W. C., and Hogeboom, G. H., "The Isolation of Cell Components by Differential Centrifugation: A Review", Cancer Res. 11: 1 (1951).

59. Bensley, R. R., and Hoerr, N., Anat. Rec. 60: 449 (1934).

60. Hogeboom, G. A., "Fractionation of Cell Components of Animal Tissues", p. 16, Methods in Enzymology I, Academic Press, New York (1955).

61. Claude, A., J. Exp. Med. 80: 19 (1944); 84: 51 (1946).

62. Beyer, K. F., and Samuels, L. T., J. Biol. Chem. (In press).

63. Hübener, J., Personal communication in Biochemistry Seminar, University of Utah, September 20, 1955.

64. LePage, G. A., and Schneider, W. C., J. Biol. Chem. 176: 1021 (1948).

65. Danby, M., Endocrinology 27: 236 (1940).

66. Srere, P. A., Chaikoff, I. L., Treitman, S. S., and Burstein, L. S., J. Biol. Chem. 182: 629 (1950).

67. Savard, K., Dorfman, R. I., and Poutasse, E., J. Clin. Endocrinol. & Metab. 12: 935 (1952).

68. Brady, R. O., J. Biol. Chem. 193: 145 (1951).

69. Hechter, O., Soloman, M. M., Zaffaroni, A., and Pincus, G., Arch. Biochem. and Biophys. 46: 201 (1953).

70. Sayers, G., and Sayers, M., Recent Progress Hormone Research II, 81 (1948).

71. Stone, D., and Hechter, O., Arch. Biochem. and Biophys. 51: 457 (1954).

72. Colonge, R. A., and Baclesse, M., Compt. rend. soc. biol., 142: 471 (1948).

73. Dorfman, R. I., Proc. Soc. Exp. Biol. and Med. 73: 223 (1950).

74. Rowinski, P., DeMure, P., and Manunta, G., Minerva mid. Vol. II, 41: 150 (1950).

75. Pfiffner, J. J., and North, H. B., J. Biol. Chem. 139: 855 (1941).

76. Butenandt, A., Naturwissenschaften 30: 4 (1942).

77. Del Greco, F., Masson, G. M. C., and Corcoran, A. C., Proc. Soc. Exp. Biol. Med. 80: 354 (1952).

78. Langecker, H., Arch. exper. Path. u. Pharmakol. 225: 309 (1955).

79. Burstein, S., Savard, K., and Dorfman, R. I., Federation Proc. 12: 185 (1953).

80. Riegel, B., Hartop, W. L., Jr., and Kittinger, J. W., Endocrinology 47: 311 (1950).

81. Gallagher, T. F., Fukushima, D. K., Barry, M. C., and Dobriner, K., Recent Progress in Hormone Research 6: 131 (1951).

82. Axelrod, L. R., and Miller, L. L., Federation Proc. 12: 173 (1953).

83. Slaunwhite, W. R. Jr., and Samuels, L. T., J. Biol. Chem. (In press).

84. Samuels, L. T., Helmreich, M. L., Lasater, M. B., and Reich, H., Science 113: 490 (1951).

85. Samuels, L. T., CIBA Foundation Colloquia on Endocrinology, VII, 180 (1953).

86. Huseby, R. A., Samuels, L. T., and Helmreich, M. L., Proc. Soc. Exp. Biol. and Med. 86: 580 (1954).

87. Ruzicka, L., and Prelog, V., Helv. Chim. Acta 26: 975 (1943).

88. Hayano, M., and Dorfman, R. I., J. Biol. Chem. 201: 175 (1953).

89. Mann, P. J. G., and Quastel, J. H., Biochem. J. 35: 502 (1941).

90. Handler, P., and Klein, J. R., J. Biol. Chem. 143: 49 (1942).

91. McIlwain, H., and Rodnight, R., Biochem. J. 45: 337 (1949).

92. Martin, R. P., and Bush, I. E., Federation Proc. 14: 252 (1955).

93. Mills, J. S., and Werner, A. E. A., Nature 169: 1064 (1952).

94. Kritchevsky, D., and Kirk, M. A., Arch. Biochem. and Biophys. 35: 346 (1952).

95. Haines, W. J., Recent Progress in Hormone Research 7: 255 (1952).

96. Neher, R., and Wettstein, A., Helv. Chim. Acta 35: 276 (1952).

97. Rosenkrantz, H., Arch. Biochem. and Biophys. 44: 1 (1953).

98. Schönheimer, R., J. Biol. Chem. 110: 461 (1935).

99. Allen, W. M., J. Clin. Endocrinol. 10: 71 (1950).

100. Singer, T. P., and Kearney, E. B., "Chemistry, Metabolism, and Scope of Action of the Pyridine Nucleotide Coenzymes", Advances in Enzymology 15: 79 (1954).

101. Slaunwhite, W. R. Jr., Personal communication.

102. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., Yankwich, P. F., Isotopic Carbon, p. 115-122, John Wiley & Sons, Inc., New York (1949).

103. Koenig, V. L., Melzer, F., Szego, C. M., and Samuels, L. T., J. Biol. Chem. 141: 487 (1941).

104. Samuels, L. T., Unpublished procedure.

105. Mann, T., Personal communication, September 21, 1955.

106. "Symposium on Metabolic Role of Lipoic Acid (Thioctic Acid)" Federation Proc. 13: 695 (1954).

107. Johnson, B. C., Ann. Rev. Biochem. for 1955, p. 427.

108. Schweet, R. S., Jagannathan, V., Katchman, B., Federation Proc. 10: 245 (1951).

109. Moyed, H. S., and O'Kane, D. J., Arch. Biochem. & Biophys. 39: 457 (1952).

110. Kern, M., and Racker, E., Arch. Biochem. & Biophys. 48: 235 (1954).

111. Mason, A., Wosilait, W. D., Terrell, A. J., Arch. Biochem. & Biophys. 48: 233 (1954.

112. Dawson, C. R., and Tarpley, W. B., The Enzymes II (1) p. 493 (1951).

113. Gillette, J. R., LaDu, P N., and Brodie, B. B., Abstract No. 39, Abstracts of Papers Presented to the Division of Biological Chemistry of the American Chemical Society, Minneapolis, Minn., September 11-16, 1955.

114. Adams, R., and Ulich, L. H., J. Am. Chem. Soc. 42: 599 (1920).

115. Mason, R., Master of Arts thesis, "The Identification of Compounds Formed from Acetate in Rat Testis Incubations In Vitro", University of Utah, June, 1956.

116. Plager, J. E., Personal communication, September, 1955.

117. Hechter, O., "Symposium: Cholesterol Metabolism", American Society of Biological Chemists, San Francisco, April 14, 1955.

118. Ochoa, S., Advances in Enzymology 15: 183, Interscience Publishers, New York, 1954.

119. Mayano, [illegible], and Lowman, M. I., J. Biol. Chem. 211: 227 (1954).